Tesis Doctoral | Adrián Mariño Enríquez

Nuevas Dianas Terapéuticas en Tumores del Estroma Gastrointestinal Identificadas mediante Bibliotecas Genómicas de ARN de Interferencia

Directores | Dra. Marta Mendiola Sabio y Dr. Jonathan A. Fletcher
Tutor | Dr. David Hardisson Hernáez

Universidad Autónoma de Madrid
Facultad de Medicina, Departamento de Anatomía Patológica

UAM
UNIVERSIDAD AUTONOMA
DE MADRID

Nuevas Dianas Terapéuticas en Tumores del Estroma Gastrointestinal Identificadas mediante Bibliotecas Genómicas de ARN de Interferencia

Este trabajo ha sido parcialmente financiado por la Fundación Alfonso Martín Escudero de Madrid (FUNDAME) y por la organización SARC (Sarcoma Alliance for Research Through Collaboration) de Ann Arbor, Michigan, Estados Unidos.

Impreso en Madrid, en septiembre de 2015.

NUEVAS DIANAS TERAPÉUTICAS EN TUMORES DEL ESTROMA GASTROINTESTINAL IDENTIFICADAS CON BIBLIOTECAS GENÓMICAS DE ARN DE INTERFERENCIA

Autor | Adrián Mariño Enríquez
Directores | Dra. Marta Mendiola Sabio y Dr. Jonathan A. Fletcher
Tutor | Dr. David Hardisson Hernáez

Tesis presentada para la obtención del título de Doctor en Medicina por la Universidad Autónoma de Madrid

Madrid, julio de 2015

Harvard Medical School **Brigham and Women's Hospital**

Jonathan A. Fletcher, M.D.
Associate Professor of Pathology
Associate Professor of Pediatrics
Harvard Medical School

Associate Oncologist
Dana Farber/Harvard Cancer Center

Director, Tumor Cytogenetics
Department of Pathology
75 Francis Street
Boston, Massachusetts 02115
Tel: (617) 732-7883 Fax: (617) 278-6921
E-mail: jfletcher@partners.org

Boston - August 31st, 2015

Doctoral Committee, Universidad Autónoma de Madrid

Re: Doctoral Thesis, Adrian Marino-Enriquez, MD

Dear Members of the Committee,

I am delighted to provide my strongest support for Adrian Marino-Enriquez, MD, in his candidacy for a PhD in Medicine at the Universidad Autónoma de Madrid. As an Associate Professor of Pathology and Pediatrics at Harvard Medical School, I attest that I have supervised his research project and the dissertation **"Novel Therapeutic Targets in Gastrointestinal Stromal Tumors, Discovered Through Genomic shRNA Library Screens" ("*Nuevas Dianas Terapéuticas en Tumores del Estroma Gastrointestinal Identificadas con Bibliotecas Genómicas de ARN de Interferencia*")**, which fulfills the criteria to be defended as a Doctoral Thesis.

Jonathan A. Fletcher

Dra. Marta Mendiola Sabio
Coordinadora del Biobanco e Investigadora
Grupo de Patología Molecular del Cáncer y Dianas Terapéuticas
Instituto de Investigación IdiPAZ

Dña. Marta Mendiola Sabio, Doctora en Bioquímica por la Universidad Autónoma de Madrid, Coordinadora del Biobanco e Investigadora Postdoctoral en el grupo de Patología Molecular del Cáncer y Dianas Terapéuticas en el Instituto de Investigación Biomédica Hospital Universitario La Paz, considero que el trabajo titulado **"*Nuevas Dianas Terapéuticas en Tumores del Estroma Gastrointestinal Identificadas con Bibliotecas Genómicas de ARN de Interferencia*"** elaborado por Adrián Mariño Enríquez bajo mi dirección, reúne los requisitos para su presentación como tesis doctoral en la Facultad de Medicina de la Universidad Autónoma de Madrid. A los efectos oportunos, firmo la presente en Madrid, el 30 de agosto de 2015.

Marta Mendiola Sabio

A Mónica, por todo lo que fue, lo que es, y lo que pudo haber sido.

Índice

Presentación

Los tumores del estroma gastrointestinal, conocidos como *GIST* (por sus siglas en inglés, *Gastrointestinal Stromal Tumors*), son los tumores mesenquimales más frecuentes del tracto digestivo, y se han convertido en un paradigma importante en oncología traslacional, ejemplificando mejor que cualquier otro tumor sólido el impacto que la investigación básica puede tener en el desarrollo racional de fármacos. En tan solo una década, la investigación traslacional aplicada al GIST ha permitido desarrollar hasta tres líneas terapéuticas de aplicación secuencial, culminando un proceso de desarrollo farmacológico racional sin precedentes. En la actualidad, existen tres fármacos dirigidos contra dianas terapéuticas específicas en GIST, que son eficaces y han sido aprobados para su tratamiento; estos fármacos han ampliado notablemente la calidad y la esperanza de vida de estos pacientes oncológicos.

Los GIST se originan por mutaciones activadoras en el oncogén *KIT*, un receptor tirosina quinasa cuya activación constitutiva es esencial para la proliferación y la supervivencia de las células tumorales. Este tumor es, por tanto, un modelo de la llamada *adicción oncogénica*, en el que la viabilidad de las células tumorales depende específicamente de la acción de un oncogén principal. Las versiones mutantes más frecuentes de *KIT* (con alteraciones en el exón 11 o en el exón 9) pueden ser bloqueadas por el fármaco inhibidor de tirosina quinasa imatinib (Glivec®). Este fármaco es enormemente eficaz en la mayoría de los pacientes, incluso aplicado en monoterapia, y consigue controlar la enfermedad durante 18-24 meses en pacientes en los que una resección quirúrgica con intención curativa no es posible. Muchos de los avances conseguidos con el estudio del bloqueo de KIT en GIST han permitido un avance importante en el tratamiento de otras neoplasias –como la leucemia mieloide crónica, o algunos subtipos de melanoma y de cáncer de pulmón- por lo que se considera un modelo contrastado y válido no solo para la investigación en sarcomas, sino también para otros tipos de cáncer. Además, debido a la especificidad del fármaco hacia su diana terapéutica, este tumor se considera un ejemplo prototípico de la llamada *medicina de precisión* en oncología, comparable al carcinoma de mama con amplificación de HER-2.

A pesar de los grandes avances en el tratamiento del GIST con imatinib, y las sucesivas líneas terapéuticas – sunitinib (Sutent ®) y regorafenib (Stivarga®) – el 90% de los pacientes desarrollan resistencia al tratamiento tras unos 2 años de respuesta clínica. Aunque se conocen algunos de los mecanismos de resistencia, que consisten principalmente en mutaciones secundarias en regiones concretas del gen, todavía se desconocen muchos de ellos y la forma de contrarrestarlos. El estudio de estos mecanismos de resistencia a terapias dirigidas en GIST es un área de investigación de gran

interés actualmente. El otro gran campo de interés es el desarrollo de nuevos fármacos dirigidos, con mecanismos de acción distintos a los inhibidores de tirosina quinasa pero complementarios a ellos, que permitan maximizar la respuesta y que, presumiblemente, no se verán afectados por los mismos mecanismos de resistencia.

El desarrollo de fármacos en la oncología contemporánea se realiza mediante un proceso de diseño racional, basado en la caracterización exhaustiva de los mecanismos moleculares que rigen la biología tumoral para descubrir vulnerabilidades específicas que puedan ser explotadas terapéuticamente. En este campo, las técnicas moleculares de alto rendimiento están revolucionado la investigación biomédica, al acelerar radicalmente el ritmo de descubrimiento de los mecanismos oncogénicos. A raíz de los importantes avances tecnológicos que han acompañado al desarrollo de las llamadas tecnologías de secuenciación masiva, en la actualidad es posible analizar simultáneamente gran cantidad de parámetros biológicos en paralelo, de forma más eficiente que los experimentos tradicionales, en los que cada parámetro debía analizarse por separado. Diversos diseños experimentales utilizando este tipo de técnicas permiten contrastar miles de hipótesis en un solo experimento, de forma objetiva y no sesgada, generando gran volumen de datos a una velocidad sin precedentes. Los primeros estudios utilizando estas tecnologías han generado resultados muy prometedores en campos tan diversos como el de las enfermedades infecciosas –*v.g.* interacciones patógeno-huésped en los virus de la gripe o el VIH–, las enfermedades neurodegenerativas –mecanismos patogénicos en enfermedad de Alzheimer– y, especialmente, el de la oncología –neoplasias hematológicas, melanoma, cáncer de pulmón, y cáncer de ovario, entre otros. Las librerías funcionales a escala genómica son técnicas de alto rendimiento validadas recientemente, ya sea utilizando RNA de interferencia (para análisis de pérdida de función) o vectores de expresión ("ORFs", para estudio de aumento de función). La característica diferencial de estos experimentos es que generan información funcional, informan de qué hacen los genes – en contraposición a la información estructural de las técnicas preexistentes, que informan sobre cómo son los genes – y aportan información más "cercana al fenotipo" y, por tanto, más directamente relacionada con la biología tumoral. En los formatos disponibles actualmente, estas librerías permiten estudiar simultáneamente la importancia relativa de más de 18000 genes (el genoma completo).

En este contexto se enmarca el proyecto de investigación presenstado aquí. Tratamos de identificar los mecanismos moleculares esenciales para la supervivencia y proliferación de las células tumorales, para identificar vulnerabilidades que puedan ser utilizadas como nuevas dianas terapéuticas, mediante el estudio funcional de todo el genoma en modelos celulares de GIST, utilizando librerías de shRNA.

Resumen

El objectivo principal de este proyecto de investigación es el desarrollo de nuevas estrategias terapéuticas que permitan controlar la resistencia a inhibidores de tirosina quinasa y que prolonguen la supervivencia global de los pacientes con GIST. Mediante el uso de tecnologías de última generación y modelos celulares representativos, queremos descubrir nuevas dianas terapéuticas en las células de GIST mediante el estudio sistemático de las alteraciones que subyacen la iniciación y progresión tumoral, y el desarrollo de resistencia terapéutica. Para conseguirlo, hemos desarrollado un plan de trabajo para establecer tecnologías de screening de alto rendimiento en el laboratorio del Profesor Jonathan Fletcher, en el Hospital Brigham and Women's, en colaboración con la Pataforma de Perturbaciones Genéticas del Broad Institute, ambos situados en Boston, Massachusetts.

Durante los últimos 5 años, hemos desarrollado las técnicas experimentales necesarias para interrogar de forma sistemática la función de todo el genoma en células de GIST. Hemos completado prevalidaciones, screening y experimentos de validación con dos líneas celulares de GIST con mutaciones de KIT, y hemos validado con éxito una nueva diana terapéutica, CDC37, una cochaperona de HSP90. Además hemos establecido una plataforma genómica funcional para el estudio sistemático de células de sarcoma, que nos permite examinar múltiples histotipos para descubrir vulnerabilidades específicas de cada tipo, así como vulnerabilidades biológicas compartidas entre diversos sarcomas.

Triangulando información genómica funcional generada mediante estos experimentos, con datos complementarios –como perfiles mutacionales, perfiles de expresión génica, o screenings químicos y farmacológicos– podremos desarrollar nuevas estrategias terapéuticas y maximizar el beneficio clínico obtenido por los pacientes afectados por estos tumores.

Summary

Gastrointestinal stromal tumors (GIST) are the epitome of clinically effective targeted inhibition of oncogenic driver mutations, and serve as a rational clinical model to evaluate mechanisms of oncogenic progression, new molecularly-targeted therapies, and mechanisms for drug response and resistance. Uninterrupted translational research efforts have led to the development of highly effective targeted agents which are now available to treat GIST patients and induce remarkable clinical responses. The major challenge we face at present is the emergence of secondary drug resistance, which stems from the morphologic and genetic complexity of the disease, before and after treatment with targeted agents, hindering long-term disease control.

The main goal of this research project is to design novel therapeutic strategies that will extend the long-term survival of GIST patients by overcoming TKI-resistance. Utilizing state-of-the-art technologies and biologically representative cellular models, we aim to identify targetable vulnerabilities in GIST cells by systematically characterizing the molecular deregulations that underlie GIST development, tumor progression, and development of resistance to pharmacologic inhibitors. To this end, we designed a hypothesis-driven study plan using a set of high-throughput genetic screening efforts at the laboratory of Professor Jonathan Fletcher, at Brigham and Women's Hospital, in collaboration with the Genetic Perturbations Platform at the Broad Institute, both located in Boston, MA, USA.

Over the past five years I have developed experimental approaches for comprehensive genome wide functional characterizations in GIST cells. We have completed the required prevalidations, screening, and follow-up experiments for 2 KIT mutant GIST cell lines, and we have successfully validated a potential therapeutic target in GIST, the HSP90 cochaperone CDC37. Furthermore, we have established a functional genomics-based discovery pipeline applicable to sarcoma cells that allows us to screen multiple sarcoma types to discover type-specific biologic vulnerabilities, in addition to shared biological themes. Triangulation of functional genomic data with orthogonal datasets –such as mutational profiles, copy number data, transcriptome and chemical screens- will allow us to design novel therapeutic strategies to maximize clinical benefit for patients affected by these devastating tumors.

Introduction

Gastrointestinal Stromal Tumor (GIST)

Basic epidemiology and clinicopathological features

GIST is the most common mesenchymal tumor of the gastrointestinal tract. Despite their relatively low frequency as a clinical problem, subclinical forms of GIST are much more common than traditionally appreciated [Kawanowa *et al.*, 2006; Agaimy *et al.*, 2007], and may be detected incidentally after surgical resections for unrelated diseases, or during autopsy [Agaimy *et al.*, 2008]. Microscopic GIST (measuring <1 cm) are observed in up to 35% of gastroesophageal resections; so-called minute sclerosing GIST (<5 mm) can be grossly detected in the proximal stomach in ~20% of autopsies of patients older than 50 years [Agaimy *et al.*, 2008]. Only a small fraction of these lesions will progress to cause clinical manifestations, reaching an annual incidence of around 11-15 per 100,000 people [Nilsson *et al.*, 2005]. A major unresolved question is the series of events that allow for these preclinical lesions to progress to a malignant GIST, and understanding these steps may provide opportunities for strategic therapeutic intervention.

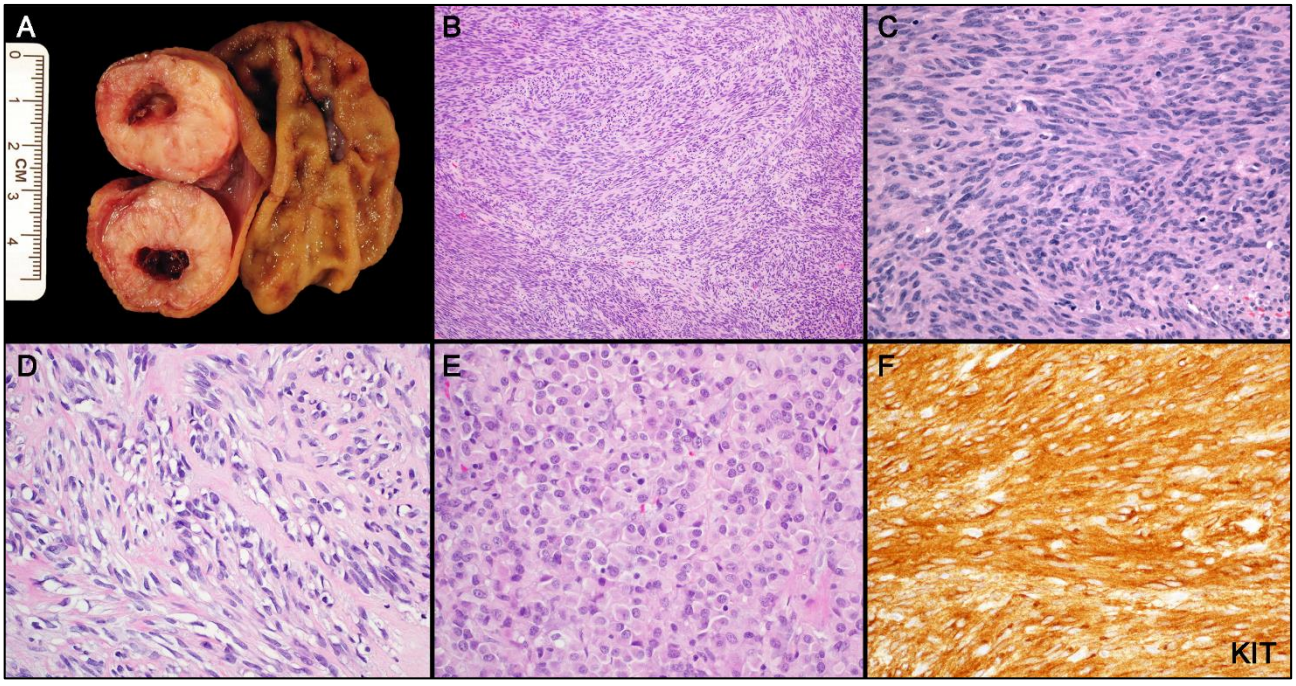
GIST typically affects older adults, manifesting at a median age of 60-65 years with no gender predilection. The most common anatomic location is the stomach (60%), followed by the small intestine, rectum, esophagus, omentum, and mesentery [Miettinen and Lasota, 2001; Miettinen and Lasota, 2006]. So-called extragastrointestinal GIST comprise tumors primarily detected in the omentum or mesentery in which an organ of origin cannot be determined. Exceptional cases are described in other locations [Long *et al.*, 2010] and may represent unusually indolent metastases from unrecognized primary tumors. The clinical presentation of GIST is rather non-specific, just related to the mass effect at the anatomic location of the tumor; submucosal lesions often cause acute gastrointestinal bleeding due to ulceration of overlying mucosa [Miettinen and Lasota, 2001; Fletcher *et al.*, 2002]. Lymph node metastases are very rare and are suggestive of a particular molecular type of GIST, which is SDH-deficient [Doyle *et al.*, 2012].

Grossly, GIST typically present as sharply demarcated tumors, showing frequently a submucosal growth (**Figure 1)**. The presence of multiple distinct tumors may represent multiple primary tumors, but most often represents local or regional metastases to the GI tract [Corless *et al.*, 2004; Gasparotto *et al.*, 2008]. Multiple GIST are occasionally syndromic: GIST can be an expression of several rare clinical syndromes in which patients are affected by multiple mesenchymal tumors, such as neurofibromatosis type 1, familial GIST, Carney-Stratakis syndrome, and Carney triad

[Nishida *et al.*, 1998; Carney and Stratakis, 2002; Miettinen *et al.*, 2006; Stratakis and Carney, 2009; Janeway *et al.*, 2011]. Syndromic presentations are much less frequent than sporadic GIST (<10% of cases), and are clinically recognizable due to particular associations. Their main relevance is that they have provided invaluable insights into the biology of the disease, pinpointing to specific genetic alterations that contribute to GIST pathogenesis.

Histologically, GIST may be composed of spindle or epithelioid cells, or show mixed cytomorphology (70, 20 and 10% of cases, respectively) (**Figure 2**) [Corless *et al.*, 2004]. Phenotype/genotype associations regarding cytomorphology are not specific enough to be clinically useful, the strongest ones being the association of epithelioid morphology and gastric location with PDGFRA mutation, and small bowel spindle cell GIST associated with neurofibromatosis. Rare morphologic appearances include cellular pleomorphism, and palisading, trabecular/endocrine, insular/epithelial, lacunar, chondroid, angiomatous or rhabdoid morphology, with no consistent clinical significance [Liegl *et al.*, 2009a; Liegl-Atzwanger *et al.*, 2010; Miettinen and Lasota, 2013; Schaefer *et al.*, 2014b]. A distinctive multinodular or plexiform growth pattern is observed in SDH-deficient GIST, in which discrete nodules of tumor cells are separated by bands of pre-existing

Figure 1: GIST morphology. Macrocopically, this lesion is a 3.5 fleshy solid mass with central necrosis and hemorrhage, located in the submucosa of the gastric wall (**A**). Histologically, the tumor consists in a homogeneous proliferation of short spindle cells (**B**) with syncytial palely eosinophilic cytoplasm, slightly fibrillary in appeareance, and ovoid nuclei with regular contours and homogeneous chromatin (**C**). Perinuclear vacuolization is commong in spindle cell GIST (**D**). About 20% of GIST show predominantly epithelioid morphology, tipically in gastric location (**E**). KIT expression, usually diffusely and strong, is readily detectable by immunohistochemistry in more than 95% of GIST.



smooth muscle, with small satellite tumor nodules [Rege *et al.*, 2011; Doyle *et al.*, 2012]. Immunohistochemically, GIST express KIT (CD117) in 95% of cases, DOG-1 in 98%, platelet-derived growth factor receptor-alpha (PDGFRA) in 80%, and CD34 in 70-80% [Medeiros *et al.*, 2004; Miettinen *et al.*, 2009; Liegl *et al.*, 2009b; Liegl-Atzwanger *et al.*, 2010]. The majority of GIST show a strong and diffuse cytoplasmic KIT staining, with a concurrent paranuclear dot ("Golgi") pattern in almost half of the cases [Hornick and Fletcher, 2007; Tabone-Eglinger *et al.*, 2008]. The small subset of GIST that do not express KIT oncoprotein (4-5% of cases) are more likely to have epithelioid cell morphology, contain *PDGFRA* oncogenic mutations, and arise in the omentum/peritoneal surface or the stomach. Notably, most KIT-negative GIST still contain imatinib-sensitive *KIT* or *PDGFRA* mutations, and similar cytogenetic profiles. Loss of normal SDHB and SDHA expression can be detected by immunohistochemistry to identify SDH-deficient GIST. GIST also express proteins indicative of a partial myogenic phenotype, including smooth muscle actin (SMA) and H-caldesmon, both present in 50-60% of cases. Desmin, however, is expressed very rarely, in less than 5% of cases (although there is substantial variation amongst the series reported) [Miettinen *et al.*, 1999; Novelli *et al.*, 2010]. So-called dedifferentiated GIST represent an exceptionally rare form of progression to a high-grade sarcoma which is by definition unrecognizable histologically in the absence of residual typical areas [Antonescu *et al.*, 2013].

Approximately 30% of GIST are malignant, and develop local recurrence or distant metastases. The biologic potential of GIST is difficult to ascertain at time of diagnosis, based solely on conventional histologic criteria; the definition of malignancy is hence based on a risk estimation system. Risk determination relies on several criteria, such as tumor size, tumor site and mitotic index, which are scored according to risk assessment scales that have been incrementally refined in large series of cases. One of the most widely accepted system is the National Comprehensive Cancer Network (NCCN) criteria from 2007 [Demetri *et al.*, 2007], which has been extensively validated for *KIT* and *PDGFRA* mutant GIST. The improved understanding of GIST biology and the continuous development of therapeutic agents with activity against specific mutations highlights the importance of mutational analysis of these tumors, which will likely be incorporated into future risk-stratification systems.

Molecular pathology and oncogenic signaling

The prototypical oncogenic driver of GIST is the receptor tyrosine kinase *KIT*, which is constitutively active due to gain-of-function mutations in ~70-80% of cases [Corless *et al.*, 2004]. An additional ~10% of GIST are driven by activating mutations in the analogous receptor kinase *PDGFRA*. PDGFRA-driven GIST show a predilection for gastric location and an epithelioid

phenotype [Heinrich *et al.*, 2003b; Wardelmann *et al.*, 2004; Corless *et al.*, 2005]. *KIT* and *PDGFRA* mutations lead to ligand-independent activation of these receptors, which activates intracellular signaling pathways controlling cell proliferation, adhesion, apoptosis, survival, and differentiation [Duensing *et al.*, 2004b]. *KIT* primary mutations usually affect exon 11 (70%), exon 9 (10%), exon 13 (1%) or exon 17 (1%), whereas *PDGFRA* mutations affect exon 18 (5%), 12 (1%) or 14 (<1%) [Corless *et al.*, 2004].

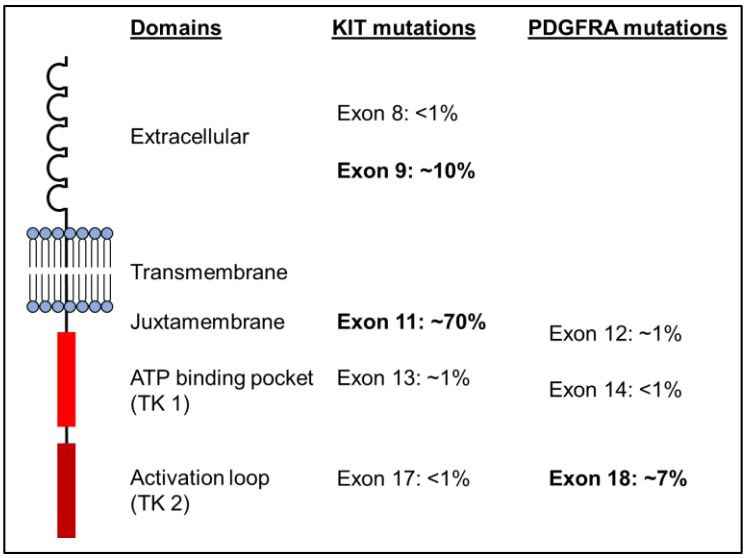


Figure 2: KIT mutations in untreated GISTs involve exons 11, 9, 13, and 17, encoding parts of the extracellular, juxtamembrane, ATP-binding pocket, and activation loop domains, respectively. PDGFRA mutations, found in <10% of GISTs, involve analogous domains.

The pattern and level of KIT expression do not correlate with the underlying mutation, although *PDGFRA*-mutants often show weaker KIT expression [Medeiros *et al.*, 2004]. *KIT* mutations in GIST not only induce receptor auto activation, but also an activation-dependent alteration of normal protein maturation and trafficking, resulting in the intracellular retention of the activated kinase within the cell; as a result, mutated KIT alleles are mainly expressed as an immature phosphorylated protein within the intracellular compartments rather than at the cell surface [Tabone-Eglinger *et al.*, 2008]. This situation is strikingly different in other RTK-driven tumors, like HER2-positive breast ductal carcinoma, in which HER2 receptors are readily accessible to blocking antibodies that bind to extracellular domains of the oncoprotein, like trastuzumab. The efficacy of similar therapeutic strategies in GIST may be limited, although recent experiments using anti-KIT monoclonal antibodies have shown promising results [Edris *et al.*, 2013]. It has also been proposed that this mechanism of cytoplasmic retention may explain the lack of correlation between imatinib response and KIT expression [Tabone-Eglinger *et al.*, 2008].

The three major signaling pathways activated by constitutive KIT and PDGFRA activation are 1) the PI3K/AKT/mTOR pathway, 2) the RAS/RAF/MAPK pathway, and 3) the JAK/STAT pathway [Duensing *et al.*, 2004b; Bauer *et al.*, 2007]. The latter pathway is known to be relevant in mast cell disease harboring *KIT* mutations but plays only a limited role in GIST. The

PI3K/AKT/mTOR and the RAS/RAF/MAPK pathways, on the other hand, are crucial for proliferation in GIST and offer potential therapeutic targets [Floris *et al.*, 2013; Patel, 2013]. To engage these downstream signaling pathways, activation of KIT is followed by binding of the adaptor protein complexes SHC, GRB2 and SOS, which transduce and amplify the signal. Downstream dependency on these two pathways is comparable to that well documented in other tumor types, such as glioblastoma [Cancer Genome Atlas Research Network, 2008], uveal melanoma [Khalili *et al.*, 2012] and non-small cell lung carcinoma [Sos *et al.*, 2009]; GIST provides a unique opportunity to explore combination therapy approaches targeting these critical pathways, given its much simpler genomic context and the excellent cellular models available.

Hyperactivation of the PI3K/AKT/mTOR pathway is central to oncogenic signaling in GIST and possibly related to imatinib-resistance, thus offering potential targets for combinatorial therapeutic strategies [Bauer *et al.*, 2007; Patel, 2013; Floris *et al.*, 2013]. AKT phosphorylation through PI3K results in increased protein translation, downregulation of the cell cycle inhibitor p27, and anti-apoptotic effects through mTOR and p70S6K [Corless *et al.*, 2011]. PTEN functions as a negative regulator of this signaling pathway. Both *PIK3CA* mutations and *PTEN* deletions have been documented in *KIT*-mutant GIST, probably contributing increased flux through this signaling pathway [Yang *et al.*, 2012; Quattrone *et al.*, 2014]. A loss or decrease of PTEN expression is observed during GIST progression, and PTEN status in GIST might be used as a possible surrogate predictive biomarker of response to PI3K inhibitors in GIST [Ricci *et al.*, 2004]. Protein kinase C θ (PKCθ), part of the PI3K/AKT/mTOR pathway, is a useful biomarker in GIST given its narrow range of expression in normal cells, including the interstitial cells of Cajal [Duensing *et al.*, 2004a; Zhu *et al.*, 2007]. PKCθ is phosphorylated in a KIT-dependent manner, either by PI3K or KIT itself, suggesting that KIT and PKCθ participate in a positive feedback loop in GIST [Ou *et al.*, 2008]. PKCθ expression and activation are crucial to GIST cell survival and proliferation regulating KIT expression, cell proliferation, and cell survival. Thus, PKCθ offers an additional potential therapeutic target in GIST, including imatinib-resistant tumors.

Activation of RAS and the downstream MAPK cascade – RAF, MEK, and ERK – ultimately results in changes in gene expression through MYC and ELK1. The activation of p90RSK by ERK increases the activity of several oncogenic transcription factors, and downregulates CIC, which is a transcriptional suppressor of ETV1 [Corless *et al.*, 2011]. Through this signaling cascade, activated KIT ultimately induces the transcription of ETV1, prolongs ETV1 protein stability and cooperates with ETV1 in tumorigenesis. The transcription factor ETV1 is a member of the ETS family that regulates the lineage-specific gene expression program characteristic of interstitial cells of Cajal and

GIST, in which it is highly expressed and plays an important role in cell growth and survival [Chi *et al.*, 2010].

A small subset of GIST are wild-type for *KIT* and *PDGFRA*, and are frequently designated "wild-type GIST". Most of these demonstrate mutations in genes like *NF-1*, *BRAF* or any of the three canonical *RAS* gene family members, which lead to activation of the RAS/MAPK pathway and add up to 10% of cases [Serrano *et al.*, 2014] (Publication II). An even smaller subset, ~5% of all GIST, demonstrate decreased expression of subunits of the SDH mitochondrial complex, most often due to loss-of-function mutations, and are grouped under the designation "SDH-deficient GIST" [Janeway *et al.*, 2011]. Finally, about 2% of GIST do not demonstrate mutations in any of the known potential driver genes and express normal levels of all SDH subunits, lacking an identifiable genetic driver. The degree of biologic overlap between different molecular subtypes of GIST is variable. In contrast with the tremendous progress in our understanding of *KIT*-mutant GIST, research in other molecular types is hindered by the lack of appropriate laboratory models.

Molecular mechanisms of tumor progression

GIST originate from interstitial cells of Cajal (ICC) or their precursors. A model of progression can be devised in which the initiating oncogenic event, most often a gain-of-function mutation of *KIT* or *PDGFRA*, results in increased proliferation that sequentially leads to ICC hyperplasia, microscopic GIST, clinical localized GIST, and metastatic GIST – ultimately followed by TKI-resistant GIST, if the patient receives TKI treatment. Such a progression model, comparable to the adenoma-carcinoma sequence that underlies epithelial neoplasia, sets GIST apart from most types of sarcomas, in which a precancerous lesion has not been identified. Key genetic events along this neoplastic sequence have been elucidated. The discrepancy in frequency between microscopic and clinical GIST indicates that GIST oncogenic progression can be arrested at several stages, so that early tumors present in up to 30% of the population regularly fail to acquire the genetic hits required to fully progress to malignancy.

KIT and *PDGFRA* mutations are initiating events in GIST tumorigenesis, but do not necessarily lead to further genetic progression or aggressive behavior; these aberrations can be found in indolent lesions such as incidental microscopic GIST [Corless *et al.*, 2004; Agaimy *et al.*, 2007]. The genetic progression of GIST beyond *KIT* mutations remains obscure, and the boundaries between focal hyperplasia of interstitial cells of Cajal and early neoplasia are unclear. Depending on their anatomic location, microscopic GIST may originate from heterogeneous subsets of interstitial cells

of Cajal with varying histopathological and molecular characteristics and different biological potential.

Most of the information regarding GIST progression is based on low-resolution cytogenetic studies: many early GIST lesions show chromosomal aberrations such as loss of 14q and occasional losses of 1p, 22q, and 15q, intuitively considered indicators of a stepwise progression. Losses of 1p and 22q, may indicate transition to an unstable karyotype and higher potential for malignancy. Despite the identification of minimally overlapping regions in these chromosomes, discovering the specific gene targets involved in crucial steps of progression in GIST remains elusive. The application of next generation sequencing technologies has failed so far to unravel major oncogenes or tumor suppressor genes in GIST, with few exceptions, perhaps partly owed to the fact that this method is not optimal to detect small genomic deletions leading to loss-of-function of tumor suppressor genes. Further studies are ongoing to determine key events involved in tumor progression. Comparative genomic hybridization to determine chromosomal losses is generally not considered in establishing the diagnosis or performing risk stratification of GIST, given its limited accessibility. However, cytogenetic data may provide helpful additional information on the prognosis of GIST. Not surprisingly, GIST harboring a higher number of chromosomal aberrations (>5) are associated with a more aggressive clinical course [El-Rifai *et al.*, 2000; Chen *et al.*, 2004; Schaefer *et al.*, 2014a]. Beyond the karyotypic information, a variety of cell cycle-related aberrations are thought to contribute to GIST progression, such as loss of p16 (*CDKN2A*), RB1 or, more rarely, deletion of *TP53* [Schneider-Stock *et al.*, 2003; Haller *et al.*, 2008; Henze *et al.*, 2012]. These are most likely late events in GIST progression, contributing a more aggressive clinical behavior and unfavorable prognosis.

Recent studies from our group identified intragenic deletions of the dystrophin gene (*DMD*), located at Xp21, as a late event in GIST progression, and related to invasion and metastasis. (Publication I) *DMD* inactivation is observed in approximately 45% of myogenic cancers, including not only GIST but also leiomyosarcomas and rhabdomyosarcomas. Myogenic sarcomas with *DMD* inactivation show a higher rate of metastases as compared to early tumor stages or benign counterparts with preserved *DMD* function. Ongoing studies aim at validating dystrophin expression in GIST as a possible biomarker of malignant behavior and metastatic potential, as well as correlating expression levels with response to TKI therapy. Targeted gene therapies, already in use in patients with muscular dystrophies (type Duchenne or Becker), aim at restoring dystrophin function in muscle cell precursors. These therapeutic approaches in non-neoplastic diseases provide a compelling

opportunity to develop targeted therapies to restore or replace dystrophin function in myogenic cancers with *DMD* loss.

Therapeutic options and mechanisms of resistance

Surgery is the preferred treatment for localized GIST, with curative intent. Resection of a primary tumor with wide margins provides a 5-year disease specific free survival of ~50%. Following surgical resection, GIST tend to recur locally or spread to the peritoneum and liver [Fletcher *et al.*, 2002; Corless *et al.*, 2011]; approximately half of the tumors relapse within 5 years, either as localized local recurrence (~7%) or as metastatic disease (~47%). In such setting, medical treatment with targeted therapy is preferred, since traditional chemotherapy (cytotoxic agents) is ineffective GIST (which explains the traditional dismal prognosis of GIST in the pre-targeted treatment era). Surgical resection of oligometastatic disease and tumor debulking have not yet proven to provide therapeutic benefit in clinical trials, but such approaches are being explored in research protocols.

Imatinib mesylate has revolutionized the treatment of GIST since its introduction in 2001, and is now widely used as first-line therapy for advanced GIST [Joensuu *et al.*, 2001; Demetri *et al.*, 2002]. GIST is the prototypical example of targeted inhibition of activating driver mutations to induce major clinical benefit for cancer patients. The preclinical demonstration of imatinib response in GIST cells, achieved in Dr. Fletcher's laboratory, led to rapid clinical translation to treat patients with metastatic GIST, most of whom had major clinical responses [Demetri *et al.*, 2002]; multicenter collaborative efforts provided the basis for regulatory approval of imatinib to treat advanced GIST [Heinrich *et al.*, 2008b; Dematteo *et al.*, 2009]. In these studies it was found that certain mutation subsets, e.g. *KIT* exon 11 mutations, respond particularly well to imatinib [Heinrich *et al.*, 2003a]. After regulatory approval, imatinib treatment remarkably improved outcomes in GIST to the current median overall survival of 5 years for patients with metastatic GIST, compared to only 19 months in the pre-imatinib era [Dematteo *et al.*, 2000; Blanke *et al.*, 2008]. Approximately 80% patients with metastatic GIST initially respond to therapy (50% partial response, 30% stable disease) resulting in a 3-year survival rate of 69–74% [Verweij *et al.*, 2004]. Primary drug resistance to imatinib mostly results from *PDGFRA* D842V point mutations or *KIT/PDGFRA* wild-type status [Heinrich *et al.*, 2008b]. The subset of tumors with *KIT* exon 9 mutations are less sensitive to standard dose of imatinib, but dose escalation to 800 mg/d provides efficient disease control. Primary resistance due to hyperactivation of signaling effectors downstream KIT is possible, but much less common and probably reflects the need of activation of several signaling pathways by independent mechanisms (Publication II). In addition, even in patients with near-complete initial response to imatinib, there are invariably viable residual GIST cells, including drug-resistant subclones which subsequently

manifest as clinical progression [Heinrich *et al.*, 2006]. In fact, 40-50% of GIST patients initially responding to imatinib develop secondary resistance within 2 years of therapy, which results from either secondary mutation in the ATP-binding pocket (exon 13-15), or the kinase activation loop (exons 17 or 18 of *KIT* and exon 18 of *PDGFRA*) in the majority of cases (>50%), or, more rarely, *KIT*/*PDGFRA* genomic amplification or activation of alternative oncogenes [Fletcher *et al.*, 2003; Debiec-Rychter *et al.*, 2005; Liegl *et al.*, 2008].

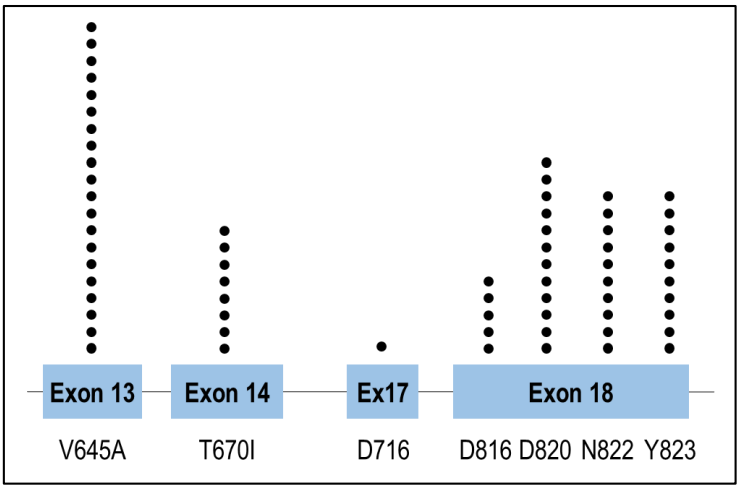


Figure 3: Frequency of KIT mutations in imatinib-treated GIST. Resistance to TKI arises most often from secondary mutations in exon 13 (encoding the ATP-binding pocket) or exon 18 (encoding the kinase activation loop). Figure adapted from [Heinrich *et al.*, 2006].

Therefore, additional anti-KIT/PDGFRA drugs were evaluated. Sunitinib malate, which inhibits a broader spectrum of tyrosine kinases than imatinib, can induce control of the clinical disease and prolong survival when given second-line following failure of imatinib [Demetri *et al.*, 2006; Heinrich *et al.*, 2008a]. This work, led by the collaborative translational and clinical team at Dana-Farber Cancer Institute/Harvard, served as the basis for worldwide regulatory approval of sunitinib as second-line agent for the treatment of imatinib-resistant advanced GIST. Unfortunately, response duration to sunitinib is relatively brief due to additional mutations of the kinase, typically occurring after 6-9 months of continuous sunitinib treatment [Heinrich *et al.*, 2008a]. This fact is largely explained by the heterogeneity of the secondary mutations present in GIST cells that conferred imatinib resistance in the first place: work from our lab, in close collaboration with Drs. Michael Heinrich and Chris Corless at Oregon Health and Science University, has proven that the resistance mechanisms in GIST vary between different patients, between different metastatic lesions in a given patient, and even between different areas from the same lesion, but typically involve reactivation of the KIT/PDGFRA kinase through a range of secondary resistance mutations (**Figure 3**) [Heinrich *et al.*, 2006; Desai *et al.*, 2007; Liegl *et al.*, 2008; Wang *et al.*, 2010]. Some of the imatinib-resistant secondary KIT mutations, e.g. those affecting the ATP-binding pocket of the kinase, are sensitive to sunitinib; but others – e.g. those affecting the activation loop - are intrinsically cross-resistant [Heinrich *et al.*, 2006]. Regorafenib, a fluoride derivative of sorafenib, has recently been approved for third-line treatment in GIST, as it is effective in cases with secondary sunitinib resistance and successfully targets secondary mutations in the kinase activation loop [George *et al.*, 2012; Demetri

et al., 2013]. Other multikinase inhibitors, like sorafenib (Publication III), nilotinib, dasatinib or crenolanib have shown modest activity against particular sets of mutations. The most recent compound within this group, ponatinib, has shown promising effects across a wide variety of mutations in preliminary studies [Heinrich *et al.*, 2013]. In contrast to imatinib, multi-kinase inhibitors have a broader kinase inhibition profile but at the same time entail a higher toxicity and rate of adverse effects in patients.

In addition to small molecule inhibitors, mutant KIT and PDGFRA can be potentially targeted using inhibitory chimeric antibodies. Recent studies provide evidence for the efficacy of a monoclonal anti-KIT antibody, designated SR1, to reduce cell growth in imatinib-sensitive and -resistant GIST cell lines *in vitro* and *in vivo* [Edris *et al.*, 2013]. SR1 treatment reduces cell-surface KIT expression, suggesting an antibody-induced KIT down-regulation to inhibit cell growth. Furthermore, SR1 induces phagocytosis of GIST cells by macrophages, indicating that SR1 may act as an opsonin and enhance immune cell-mediated tumor clearance [Edris *et al.*, 2013].

The structural heterogeneity of different KIT/PDGFRA oncoproteins complicates the design of drugs that could effectively bind to these mutant RTKs and inhibit the oncogenic signal in an efficient and specific manner. Indirect mechanisms to target KIT and PDGFRA oncoproteins may provide effective systems to overcome TKI resistance. KIT/PDGFRA signaling in GIST cells is exquisitely dependent on the chaperoning function of HSP90, which is required for folding, localization and stabilization of the mutant oncoproteins. Preclinical validations have shown compelling responses to HSP90 inhibition in GIST, *in vitro* and *in vivo*: after HSP90 inhibition by a variety of compounds, KIT oncoproteins are rapidly degraded, with antiproliferative and pro-apoptotic consequences. Clinical translation of HSP90 targeting has been challenging, presumably because inhibition of HSP90 also targets HSP90-dependent, non-oncogenic, client proteins limiting the tolerance to sustained potent HSP90 inhibition. Initial clinical trials of ansamycin-analogue HSP90 inhibition in GIST have shown evidence of biological activity, although they achieved relatively low response rates and substantial toxicity [Wagner *et al.*, 2013].

Besides direct or indirect targeting of KIT/PDGFRA, targeting the signaling pathways downstream of KIT/PDGFRA is a potentially effective and attractive strategy to by-pass the mutational heterogeneity of the oncogenic driver. Previous work from our lab has shown that most KIT/PDGFRA oncoproteins in GIST share common downstream signaling mechanisms, irrespective of their particular mutations and whether these are TKI-sensitive or TKI-resistant [Duensing *et al.*, 2004b]. Two main molecular pathways transduce the oncogenic signal downstream of KIT/PDGFRA and regulate proliferation and survival in GIST: the PI3K/AKT/mTOR pathway, and the

MEK/MAPK pathway, activated by GRB2/RAS/RAF mechanisms [Duensing *et al.*, 2004b; Bauer *et al.*, 2007; Zhu *et al.*, 2007]. PI3K inhibitors LY294002 and GDC-0941 targeting *PI3K* mutations effectively decrease proliferation and induce cell death in various GIST cell lines in preclinical studies *in vitro* and *in vivo* [Floris *et al.*, 2013]. Inhibition of PI3K by LY294002 substantially inhibits AKT, S6, and 4EBP-1 phosphorylation in GIST cell lines with PI3K and KIT expression levels remaining constant. Inhibition of mTOR has shown limited success, which has been ascribed to the activation of AKT that occurs following mTORC1 inhibition. Further drugs targeting the PI3K/AKT/mTOR pathway are currently being investigated in clinical trials in combination with imatinib, including the KIT/PI3K inhibitor BKM120, KIT/mTOR inhibitor everolimus, the mTOR inhibitor temsirolimus, and the KIT/AKT inhibitor perifosine [Schoffski *et al.*, 2010]. Activation of RAS/RAF/MAPK signaling pathway may be targeted with MEK or BRAF inhibitors, such as trametinib or vemurafenib, currently used to treat BRAF-mutant melanoma. So far, only one GIST patient with a *BRAF* V600E mutation successfully treated with the ATP-competitive BRAF inhibitor dabrafenib has been reported [Falchook *et al.*, 2013]. Given the activation of PI3K and MAPK pathways downstream KIT, it is not unexpected that dual inhibition of both pathways results in arrested proliferation and cell death in the preclinical setting. Toxicity of such combinations is still too high for them to be considered realistic options in the clinic, but novel compounds or improved administration regimens should enable dual downstream targeting.

Understanding the molecular mechanisms of response and resistance to TKI therapy in GIST generates invaluable biologic insights, applicable to other tumor types driven by distinct oncogenic mutations. The mechanisms of resistance in malignancies as diverse as chronic myelogenous leukemia, EGFR- or ALK-driven non-small cell lung carcinoma, HER2-driven breast carcinoma or melanoma, are remarkably similar to those observed in GIST. Several features make GIST an ideal model for designing innovative therapeutic approaches to overcome drug resistance: first, the absolute addiction of GIST cells to sustained signaling from the KIT or PDGFRA oncogenic driver, with known signaling pathways that provide useful reference points. Second, the remarkably simple and stable genomic landscape characteristic of GIST. Third, the availability of unique cellular models that accurately represent the clinical reality. Finally, the availability of drugs and tool compounds with distinct specificity profiles, which provide unique tools to effectively modulate and study essential signaling pathways. In fact, GIST research has pioneered the development of targeted therapies, and successful translational efforts have led to the sequential approval of three lines of treatment, with highly effective targeted compounds that have had unmatched impact in patient survival. GIST provides an unprecedented opportunity to study in the laboratory the mechanisms of

drug response and resistance, combining information generated in the clinic and modeling innovative approaches that can be tested with greatest likelihood of clinical translation.

Functional genetic screens

An enhanced understanding of the signaling networks and the molecular mechanisms of growth and survival in tumor cells, and in GIST cells in particular, will allow us to rationally develop additional therapeutic strategies, with maximal likelihood of prolonged clinical success. The addiction of GIST cells to their KIT/PDGFRA oncogenic drivers imposes secondary dependencies on genes that are not oncogenes in and of themselves, but which can be inhibited therapeutically in GIST as KIT/PDGFRA oncogene-specific "synthetic lethal" strategies [Scholl *et al.*, 2009]. As a consequence, targeting KIT/PDGFRA or its downstream effectors will likely remain the main focus of GIST therapy, but additional targets will be exposed if the key nodes and vulnerabilities resulting from the oncogenic status can be discovered. Several high-throughput technologies allow the interrogation of multiple variables in a multiplexed manner in cancer cells; the most common and available of such techniques is the systematic characterization of cancer cells genome –as illustrated by the profusion of genomic studies, spearheaded by consortium efforts like The Cancer Genome Atlas, TCGA. Such genomic annotations reveal the genetic make-up of cancer cells, including mutational load and copy number information; cataloging cancer mutations offers critical insights into the biology of malignancy, based in structural information (i.e. integrity of genes or their abnormalities). However, to understand the function of the cancer genome and the genetic dependencies of cancer cells, a complementary set of functional genomic approaches is required. Descriptive information of additional cellular "-omes" provide complementary angles (such as transcriptome, proteome, metabolome or secretome), while functional techniques allow for experimental manipulations at a genomic scale (**Figure 4**).

Functional genomic techniques aim to characterize the functional relevance of individual genes in relationship to a given phenotype. Instead of analyzing if a gene sequence or dosage is normal or abnormal in a cancer cell, functional approaches provide information regarding the relative importance of each gene for the cancer cells to accomplish certain cellular functions, such as survive, proliferate, invade, or metabolize a given compound. This approach is interventional, rather than descriptive, since the cells are subject to genetic manipulations and then closely followed to document the resulting phenotype. Functional genetic manipulations have been utilized for decades in molecular biology, but recent technological advances, combined with a better understanding of some molecular

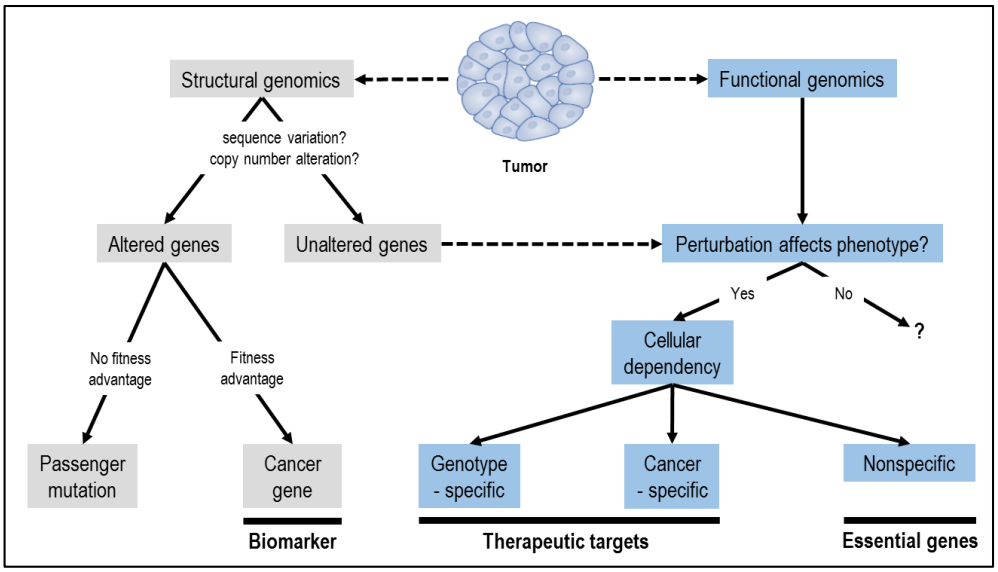


Figure 4: Systematic genomic approaches to study cancer biology. The impact of functional genomic screens is maximized by the integration with orthogonal technologies like genome characterization by massively parallel sequiencing and copny number analysis. Figure adapted from [Boehm and Hahn, 2011].

processes (such as gene silencing through RNAi) have enabled highly-parallel multiplexing of genetic perturbations. Two complementary approaches can be used for functional genetic experiments: loss-of-function assays, performing RNAi-mediated gene knockdown with shRNAs (short-hairpin RNAs) [Luo *et al.*, 2008] or Cas9-mediated knockout with sgRNAs (short guide RNAs) [Shalem *et al.*, 2014]; and gain-of-function experiments, inducing high-throughput ORF-mediated gene expression utilizing lentiviral expression vectors [Johannessen *et al.*, 2010; Yang *et al.*, 2011]. Collections of reagents have been developed in the form of libraries that enable genetic perturbations at a genome-wide scale. One such collection is The RNAi Consortium (TRC) developed at the Broad Institute, a biomedical research institute located in Boston (Massachusetts, USA) and founded as a collaboration between Harvard University and the Massachusetts Institute of Technology. The TRC library hosted at the Genetic Perturbations Platform of the Broad Institute consists of shRNA, sgRNA and ORF (open reading frame) libraries that cover the entire human and mouse genomes.

To identify cellular dependencies and the underlying responsible genes, either involved in essential GIST growth/survival pathways or in drug resistance, we performed genome-wide functional evaluations using the TRC libraries. The experiments described in this work correspond to pooled loss-of-function shRNA approaches, given that the ORF and sgRNA libraries were not fully developed at the beginning of this project (although more recent experiments within our group utilize sgRNA and ORF libraries).

The technology of pooled shRNA libraries was developed to perform unbiased comprehensive loss-of-function genetic screens and allows for the systematic correlation of gene knockdowns with a given cellular phenotype in a high-throughput manner [Cheung *et al.*, 2011]. Each shRNA, a synthetic double-stranded RNA of approximately 21nt, triggers degradation of a cellular messenger RNA, which is cleaved by a protein of the Argonaute family within the RNA-induced silencing complex. The extent to which the target mRNA is degraded is variable, and is referred to as the 'knockdown efficiency' for each particular shRNA. The specificity of shRNAs for mRNA targets is also variable, and off-target effects are common events in RNAi experiments (see Discussion). Given that any single shRNA may function in both on- and off-target pathways, proper interpretation of shRNA experiments requires that multiple independent shRNA targeting the same gene result in the same phenotype. shRNA libraries are hence redundant and incorporate multiple shRNAs per gene. In its current iteration, the TRC shRNA library consists of 98,000 shRNA constructs corresponding to 18,000 human genes, with ~6 non-overlapping hairpins per gene, which can be delivered to the cells of interest in a single pooled lentiviral infection. The shRNAs are introduced into retroviral vectors through conventional cloning methods; in its lentiviral form, the DNA sequence of the perturbation is delivered into the genome of the host cell, resulting in a permanent, heritable genomic modification that leads to constitutive expression of shRNAs in the host cell.

Pooled screening assays may follow two general experimental designs: positive or negative selection screens, depending on the phenotype under consideration. The simplest example of a negative selection phenotype is cell death: perturbations that cause cells to die are depleted from the population over time. Such an approach has been used to identify genes that are broadly essential to cell viability, as well as to identify essential genes to specific cell types [Cheung *et al.*, 2011]. A large-scale approach using this methodology is underway in an effort called Project Achilles, which aims to systematically characterize cancer cell vulnerabilities for a wide variety of tumor types. The Project Achilles dataset is publicly available online (http://www.broadinstitute.org/achilles) and to date includes data on 216 cell lines as described in a recent publication by Glenn Cowley and collaborators [Cowley *et al.*, 2014]. A limiting characteristic of negative selection assays is that a perturbation can only be depleted to zero, from the starting level at which it was present in the original pool. This results in limited signal-to-noise ratio for negative selection screens with large libraries, compared to smaller sub-libraries. Positive selection screens focus instead on cell survival, relying on overrepresentation of a subset of cells in response to a given perturbagen in the population; therefore, they have a much larger dynamic range. Examples of positive selection screens include viability assays to rescue cells from a toxic intervention, such as a pharmacologic inhibitor, or detection of specific markers induced by transcriptional activators [Wilson *et al.*, 2015].

The design of the experiments described in this work, in brief, is as follows: 200-300 million GIST cells are infected in a single pooled infection with the complete shRNA library, for a representation of 200-300 cells per shRNA. Infected cells are selected with puromycin, and six experimental replicates are set up in parallel. The cells are then expanded under different selective conditions (untreated or under pharmacologic inhibition of KIT/PDGFRA) and after ~16 doublings the representation of the initial shRNA library is assessed by genomic DNA extraction, sequencing, and quantification of the relative proportion of hairpins in the surviving cells (**Figure 5**). Several computational algorithms are applied to collapse information from multiple hairpins to the corresponding genes, the resulting output being a bidirectional rank of genes according to depletion or enrichment in the final population. This conversion of hairpin-level data to gene-based information is complex and relies on several computational algorithms that have been developed as our understanding of the RNA interference process improved. shRNA reagents induce different degrees of on-target and off-target effects, and gene suppression resulting from both sources may contribute to the phenotype of interest. The use of different computational approaches allows for a good discrimination of on-target effects, or at least provides a level of confidence in the results that can be used before embarking on downstream validation experiments (see Discussion section). Once several analyses are performed, the definition of a "hit" may be controversial, but is usually established in statistical terms based on false discovery rate and p-value thresholds. We focused on the extremes of the distribution (top 1%, top 5%, and top 10% of ranked genes), but the signal-to-noise ratio in some of the analyses suggests that the gene ranks provide valid information also for less extreme values. In any case, follow-up confirmatory experiments are performed with independent shRNAs and other reagents to validate the results.

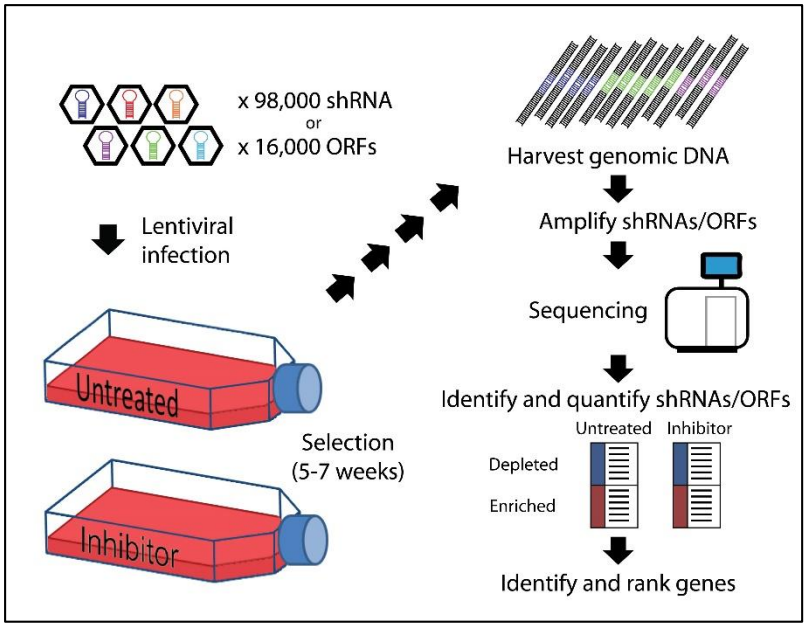


Figure 5: Experimental design for shRNA pooled screens to identify essential genes, specific cell dependencies, and synthetic lethal interactions in GIST.

A complementary aspect to shRNA pooled library screens is the high-throughput gain-of-function screening technology, currently at an earlier stage of development. A lentiviral expression

library created at the Broad Institute comprises 18,000 distinct ORFs mapping to ~16,000 genes [Yang *et al.*, 2011]. Positive selection ORF screens are particularly suited for the study of resistance to pharmacologic inhibitors. Specific challenges of this experimental design include the difficulty to generate physiologic levels of expression, and the limited representation of transcript variants. Nevertheless, the juxtaposition of gain-of-function data to our GIST shRNA screening datasets will maximize opportunities to identify crucial biologic pathways, i.e. targets and pathways that are convincingly identified as crucial regulators of GIST viability in both the loss-of-function and gain-of-function evaluations.

Hypotheses and Specific Aims

Previous studies have highlighted that Gastrointestinal Stromal Tumors (GIST) provide compelling opportunities to target oncogenic driver mutations. Most GIST depend upon continuous signaling through mutant constitutively-activated forms of KIT or PDGFRA, and biologically credible *in vitro* GIST models have enabled efficient, highly-informative validations of therapeutic strategies relevant to the mutant KIT/PDGFRA kinase pathways. Targeted KIT/PDGFRA inhibition with small molecules like imatinib has profound effects on GIST viability and growth and, accordingly, most GIST patients exhibit remarkable clinical responses upon treatment with such inhibitors. However, ~90% of clinically advanced GIST are composed of heterogeneous populations of cells that harbor a range of imatinib-resistant secondary KIT/PDGFRA mutations, which cannot be durably suppressed by any given KIT/PDGFRA inhibitor and eventually lead to clinical progression. Importantly, the genetically diverse KIT/PDGFRA oncoproteins, irrespective of their variable sensitivity to tyrosine kinase inhibitors, share common downstream signaling mechanisms in GIST cells, including activation of the MEK/MAPK and PI3K/AKT/mTOR pathways. We hypothesize that a nuanced understanding of these constitutively activated signal transduction pathways will uncover vulnerabilities that can serve as biologically rational targets for combination therapies to maximize the clinical benefit of KIT/PDGFRA inhibition. With this objective in mind, we seek to expand the current knowledge of GIST biology and to design effective therapeutic strategies that will overcome TKI resistance in GIST patients.

The overarching goal of this project was to improve the long term survival of GIST patients by designing novel efficient combination therapies. To this end, we performed functional studies of GIST cells for target discovery and preclinical validations of optimal GIST therapeutic strategies according to a research plan with the following specific aims:

Aim 1: To establish a functional genomics platform to discover novel gene dependencies and biologic vulnerabilities in sarcoma cells. With the goal of systematically interrogating functional dependencies on a genome-wide scale in patient-derived cellular models of sarcoma, we established a working collaboration with the Genetic Perturbation Platform at the Broad Institute of MIT and Harvard. We customized the experimental conditions to optimally evaluate large scale sarcoma cultures, both for loss-of-function pooled screening as well as, on a second phase, for gain-of-function arrayed experiments. The reagents and infections were performed at the Broad Institute, while large-scale cell culture expansion took place at Brigham and Women’s Hospital.

Aim 2: To characterize essential genes and pathways involved in GIST proliferation and survival. To identify essential genes in GIST, we performed loss-of-function genome-scale shRNA screens in three GIST cell lines, using pooled lentiviral libraries (54K shRNAs, targeting 11K genes) within the RNAi Consortium at the Broad Institute of MIT and Harvard. We have validated the highest scoring leads, with follow-up individual functional assays and pharmacological inhibitors. In ongoing experiments, we are undertaking a complementary but orthogonal approach, in which gain-of-function screens will be performed in the same GIST lines using novel ~16K cDNA open reading frame (ORF) expression libraries.

Aim 3: To identify mechanisms of drug response and resistance, and discover synthetic lethal interactions with KIT/PDGFRA inhibition in GIST cells. The Aim 2 genome-scale screens will be leveraged by modifier screens performed with IC50 doses of KIT/PDGFRAi (imatinib and sunitinib). In these studies we will identify targets whose suppression synergizes with KIT/PDGFRA inhibition, as well as genes whose expression rescues the cells by conferring resistance to treatment. The goal is to identify effective combination therapies for GIST.

Aim 4: To validate novel rationally-designed targeted therapeutic strategies for GIST. The functional datasets generated by the Aim 2 and Aim 3 high-throughput screens will be leveraged by our parallel sequencing studies in GIST (exome and whole genome sequencing, transcriptome sequencing). To maximize the insights from these datasets, we will triangulate the genomic, expression profiling and functional data to prioritize potential targets for preclinical validations. The requisite bioinformatic analysis will be accomplished with assistance from our computational biology team at the Broad Institute, and the preclinical validations will be performed using knockdown methods and selective pharmacologic inhibitors of the priority targets.

The results presented in this dissertation correspond to the completion of Aims 1 and 2 of this large, multi-institutional project, which has been developed over the last 5 years. Aims 3 and 4 are being pursued in ongoing experiments, and will likely take several years for completion. Within this highly translational research work, we are utilizing innovative technologies to systematically characterize cellular models of GIST that accurately represent the clinical disease. Collectively, these studies will foster the development of urgently needed effective combination therapies by identifying vulnerabilities and synthetic lethal interactions in GIST cells. In addition, the mechanistic insights resulting from these studies will be relevant to other kinase-dependent sarcomas.

Material and Methods

Cell lines

GIST882 is an imatinib-sensitive human cell line established from an untreated GIST with a primary homozygous missense mutation in *KIT* exon 13, encoding a K642E mutant KIT oncoprotein [Bauer *et al.*, 2006]. GIST-T1 is an imatinib-sensitive GIST cell line established from an untreated metastatic GIST containing a homozygous 57 bp deletion in KIT exon 11 [Taguchi *et al.*, 2002]. GIST430 and GIST48 are KIT exon 11 mutant GISTs established from patients progressing clinically on imatinib; these, respectively, have V654A ATP-binding pocket and D820A activation loop secondary imatinib-resistance mutations [Bauer *et al.*, 2006]. GIST48B is a subline of GIST48 which, despite retaining the activating KIT mutation in all cells, expresses essentially undetectable levels of KIT transcript and protein. GIST882 and GIST-T1 cells were maintained in RPMI 1640 with 15% fetal bovine serum (FBS) supplemented with penicillin/streptomycin and 1% (v/v) L-glutamine. GIST48 and GIST430 were maintained in Ham's F10 culture medium containing 15% FBS, penicillin/streptomycin, L-glutamine, Mitotracker+, and bovine pituitary extract. All GIST lines used in this study were cultured from highly-credentialed master stocks (*KIT* mutation sequencing authentications and 250K *NspI* SNP whole-genome profiles) for no more than three months before being used in the assays. HEK293T cells obtained from ATCC were used to produce lentiviral constructs.

Primary shRNA pooled screen

Development and applications of the 54K lentiviral shRNA pooled library from The RNAi Consortium (TRC) have been described recently in the literature [Cheung *et al.*, 2011]. For this particular screen, GIST-T1 and GIST882 cells were infected with a pool of 54,020 pLKO.1-shRNA-encoding lentiviruses targeting 11, 194 genes at a multiplicity of infection of ~0.3 and subjected to puromycin selection (2 μg/mL) for 5 days. Eight (GIST-T1) and six (GIST882) replicates of ~20 million cells were established post-selection and plated in T175 flasks, fed on a 3-times-per-week schedule and passed at confluence, approximately weekly. For every passage, a minimum of 12x10E6 cells was passed into new flasks for continued culture and expansion over 6-7 weeks, while the remaining cells were harvested by centrifugation, resuspended in 0.5 ml PBS, and stored at -20ºC for subsequent genomic DNA extraction and analysis. Final harvests of the infected cells were used for genomic analysis according to the previously validated methods: genomic DNA was isolated from cell pellets by digestion with proteinase K followed by isopropanol precipitation. To amplify the shRNAs encoded in the genomic DNA, PCR was performed for 33 cycles at an annealing temperature

of 66°C using 2–6μg of genomic DNA, a barcoded primer pair, and DNA polymerase. So that PCR products obtained from different samples could be sequenced together, forward primers containing unique 2-nucleotide barcodes were used [Barcoded forward primer: AATGATACGGCACCACCG AGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAANNGACGAAAC (N indicates location of sample-specific barcode sequence). Common reverse primer: CAAGCAGAAGA CGGCATACGAGCTCTTCCGATCTTGTGGATGAATACTGCCATTTGTCTCGAGGTC]. After purifycation, the PCR products were quantified by ethidium bromide staining after gel electrophoresis, pooled at equal proportions, and analyzed by high-throughput sequencing (Illumina) using this primer: AGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAA. Sequencing reads were deconvoluted using GNU Octave software by segregating the sequencing data by barcode and matching the shRNA stem sequences to those expected to be present in the shRNA pool, allowing for mismatches of up to 3 nucleotides. The 54,020 shRNAs were ranked by their relative depletion from the cell pool, and the corresponding 11, 194 genes, were then scored according to the rank of the second-most depleted shRNA (out of typically 5 shRNAs targeting each gene). The in-house-developed GENE-E program was used to rank the genes according to the second best-scoring shRNA within each hairpin set (http://www.broadinstitute.org/cancer/software /GENE-E/download.html).

Preparation of lentiviral shRNA constructs for validations

Lentiviral constructs encoding shRNA specific sequences targeting *CDC37* and *KIT* gene transcripts on the pLKO.1puro backbone were selected from the TRC library (TRCN0000116632-TRCN0000116636 and TRCN0000000388- TRCN0000000392; the TRC website is http://www.broadinstitute.org/rnai/trc/lib). Lentivirus preparations were produced by lipofectamine-mediated cotransfection into HEK293T cells of pLKO.1puro containing empty vector or CDC37/KIT-specific shRNA, and helper virus packaging plasmids Δ8.9 and VSV-G (at a 10:10:1 ratio). Culture supernatants containing lentivirus were collected 24, 36, 48 and 60 h post-transfection. Viral preparations were pooled and stored at -80°C. Well-validated shRNA providing >90% knockdown were used for CDC37 knockdown in follow-up experiments (TRCN0000116632: clone 1; and TRCN0000116633: clone 2).

Validation cell culture and lentiviral infections

GIST cells were seeded in 6-well plates and lentiviral infections were performed overnight in the presence of 8 μg/mL polybrene. Following infection, shRNA-expressing cells were selected with 2 μg/mL puromycin. GIST882 and GIST-T1 cells were maintained in RPMI 1640 with 15% fetal bovine serum (FBS) supplemented with penicillin/streptomycin and 1% (v/v) L-glutamine. GIST48

and GIST430 were maintained in Ham's F10 culture medium containing 15% FBS, penicillin/streptomycin, L-glutamine, Mitotracker+, and bovine pituitary extract. Cell culture images by bright field microscopy were obtained using SPOT software (SPOT Imaging Solutions, Sterling Heights, MI, USA) and an Eclipse TE2000-5 inverted microscope (Nikon, Japan). Cells were lysed for western blot analysis at 4, 10 and 20 days post-infection.

Antibodies and other reagents

Antibodies for immunoprecipitation were to KIT (sc-13508; Santa Cruz Biotechnology) and CDC37 (ab2800; Abcam). Antibodies for immunoblotting were KIT (A4502; Dako), CDC37 (ab2800; Abcam), phospho-KIT Y721 (3391s; Cell Signaling Technologies), phospho-AKT S473, total AKT and phospho-MAPK T202/T204 (9271L; 9272 and 9101L, respectively; Cell Signaling Technologies), MAPK (61-7400; Invitrogen), and actin (A4700; Sigma-Aldrich). Lipofectamine™ and Plus Reagent™ were from Invitrogen. Puromycin and polybrene were from Sigma-Aldrich.

Western blotting and immunoprecipitation

Whole cell lysates were prepared from cell line monolayers in lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium orthovanadate) containing protease inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Electrophoresis and immunoblotting were carried out as described previously [Rubin *et al.*, 2001]. Detection was by chemiluminescence (Immobilon Western, Millipore Corporation), with image capture by a FUJI LAS1000-plus chemiluminescence system. KIT and CDC37 immunoprecipitations were performed using Sepharose-protein G beads (Invitrogen).

Cell cycle analysis

Analyses were performed 10 days after lentiviral infections with puromycin selection. GIST882, GIST-T1 and GIST430 cells in 6-well plates were trypsinized, pelleted, and then washed once with Hanks Balanced Salt Solution at room temperature. For nuclear DNA content staining, cells were fixed with 70% ethanol for 24 h. For nuclear staining, propidium iodide (PI) solution (Roche) was added to the cells and incubated for 15 minutes at 37°C. The cell suspension was analyzed in a flow cytometer (NPE Quanta, NPE Systems) and data analysis was performed using ModFit LT software 3.1 (Verity Software House, Topsham, ME).

Cell viability assays

Cell viability studies were carried out using the CellTiter-Glo luminescent assay (Promega, Madison, WI), in which luciferase-catalyzed luciferin/ATP reaction provides an indicator of cell number and metabolic activity. GIST cell lines were plated at 5,000 or 10,000 cells per well in 96-well flat-bottomed plates (Falcon, Lincoln, NJ) and cultured for 12 days post-infection in serum-containing media. The CellTiter-Glo assay luminescence was measured with a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) at days 0, 4, 8 and 12. Readings were normalized to the day 0 and pLKO control reads. All experimental points were measured in triplicate wells and replicated in at least two independent plates.

Xenograft studies

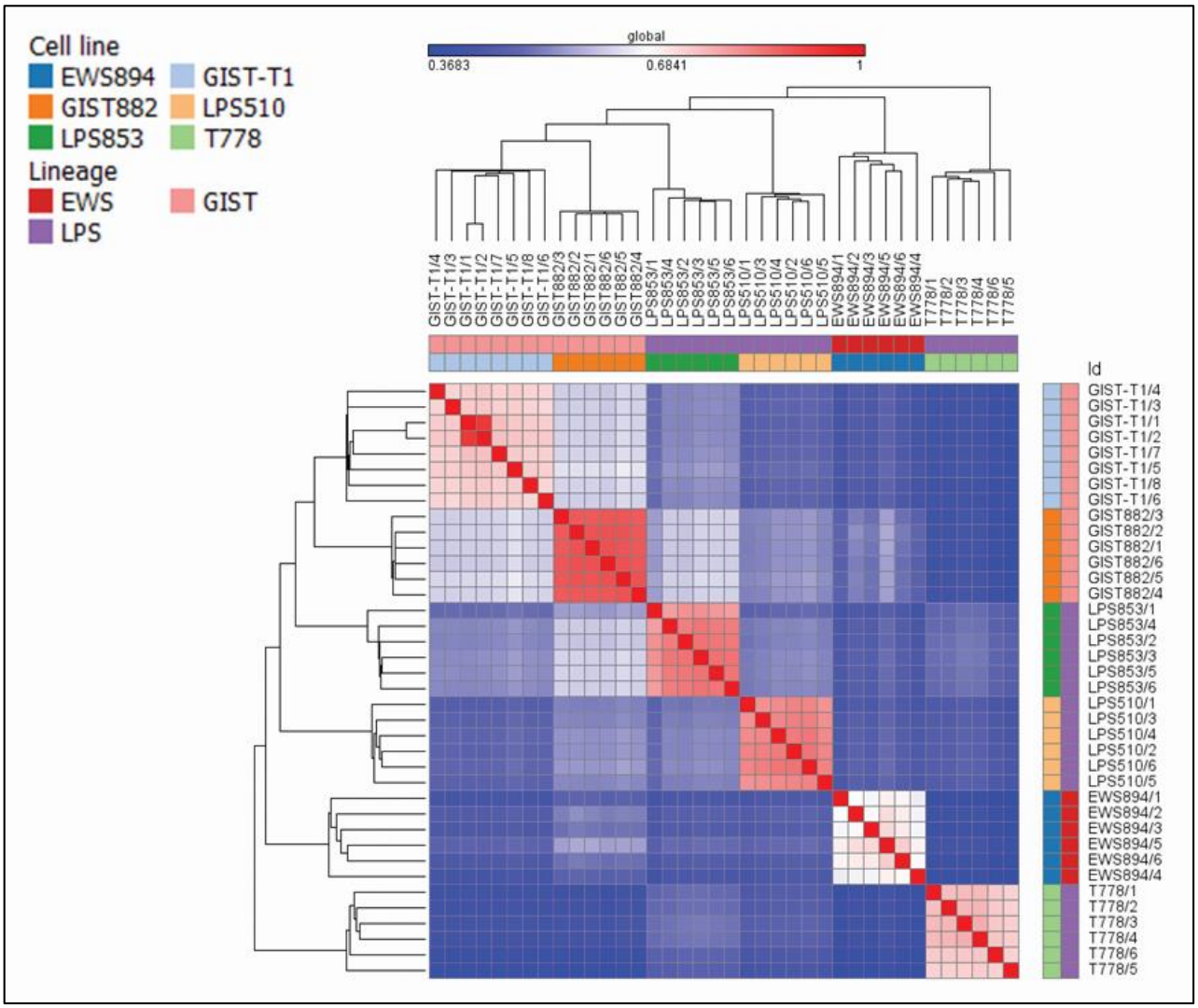
All mice used were maintained, injected and sacrificed in accordance with an approved IACUC protocol at Dana Farber Cancer Institute. Athymic nude mice were injected subcutaneously with GIST-T1 cells expressing *CDC37*-targeting shRNA in one flank (shRNA1: TRCN0000116632; shRNA 2: TRCN0000116633; n=3 each) and empty pLKO.1 lentiviral vector in the other flank (n=3). In all, 2×10^6 infected cells on puromycin selection were resuspended in Matrigel (BD Biosciences) and implanted subcutaneously at each injection site. Tumor volume was evaluated weekly. Mice were killed by CO_2 inhalation and necropsied 6 weeks after injection. Tumors were resected, measured and photographed. Whole tumor lysates were prepared for Western blot analysis (as described above), and tissue was fixed in formalin for routine histologic processing and examination.

Results

shRNA pooled library screen performance in GIST-T1 and GIST882 cells

The reproducibility of short hairpin RNA (shRNA) enrichment and depletion profiles was evaluated across the experimental replicates, as a quality control. The GIST882 and GIST-T1 screen replicates clustered closely within each cell line by both unsupervised and consensus clustering of shRNA depletion and enrichment profiles, attesting to the robustness of the screens. In addition, the GIST882 and GIST-T1 replicates clustered next to each other when compared with a reference data set of 12 publicly available cancer cell line data sets [Luo *et al.*, 2008], as well as after linkage analysis in the context of additional unpublished sarcoma cell lines (**Figure 6**). A comprehensive list of the 11,194 ranked genes, along with annotation for the shRNA clones, is provided in Table 1.

Figure 6: Similarity matrix and unsupervised hierarchical clustering of the shRNA profiles of 6 sarcoma cell lines, cultured after pooled infection with the TRC shRNA library (Pearson correlation; average linkage analysis), showing clustering of the replicates corresponding to each cell line.



CDC37 is essential for GIST cell survival

In the pooled proliferation screens, cells carrying shRNAs that targeted proliferation-essential genes were depleted from the cell population over time. Scored according to the second best-scoring shRNA within each hairpin set, 25 out of 56 genes ranked in the top 0.5% of the distribution for both GIST882 and GIST-T1 (Table 1, left column). Of these 25 genes, 19 were also within the top 0.5% in at least 8 of 12 comparison non-GIST cancer lines [Luo *et al.*, 2008], and were thus identified as 'commonly essential' genes not specific to GIST (**Figure 7**). These genes belonged to functional categories known to be essential in cancer cell lines: regulation of mRNA splicing and processing, protein translation, and ribosome and proteasome structure and function. The other six genes were selectively essential for the two GIST cell lines vs the other lines (bold italic font, Table 1 left column): five of these encode mRNA processing proteins, whereas the remaining gene, *CDC37*, encodes an HSP90 cofactor. CDC37 is known to coordinate HSP90 chaperoning activity for a subset

GIST882 and GIST-T1	GIST882 only	GIST-T1 only
CDC37	***UBC***	***PSMC4***
POLE	***DYNC1H1***	***EFTUD2***
RPS18	***VCP***	***PSMA2***
PSMA3	***HNRPK***	***PRPF3***
SFRS3	***PABPN1***	***NCBP2***
EIF3S10	***SNRPD1***	***ABCB7***
RPS13	***PSMC1***	***EIF3S3***
RPS29	***FRAP1***	***ATPBD1C***
RPL23A	***AFG3L2***	***RPAP1***
PSMB2	***LOC375133***	***RBM8A***
RPL5	***PSMA6***	***DDX48***
RPS9	***EIF3S5***	***RNPS1***
RPS17	***TSG101***	***COPS2***
RPL31	***PTPRCAP***	***RPS10***
ARCN1	*TUBB*	***MLXIP***
PHB2	*RPS19*	***ATP1A1***
RPS7	*KARS*	***POLR2F***
RPS27A	*SNRPD2*	***RUVBL2***
RPS8	*PSMA1*	***NUP205***
RPS15A	*RPS14*	***MKI67IP***
PSMD1	*HNRPU*	*RAN*
U2AF2	*RPS6*	*RPS3A*
CHD4	*RPS3*	*RPS4X*
AQR	*ASCC3L1*	*RPS11*
NHP2L1	*RPL34*	*RPL7*
	NDUFA4L2	*RPL6*
	RPSA	*EIF5B*
	USP39	*EIF2S2*
	PHB	*SNRPE*
	EIF1AX	*HSPE1*
	TPR	*U2AF1*

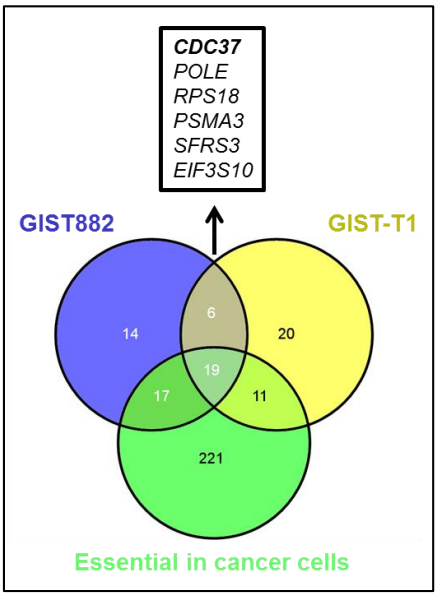
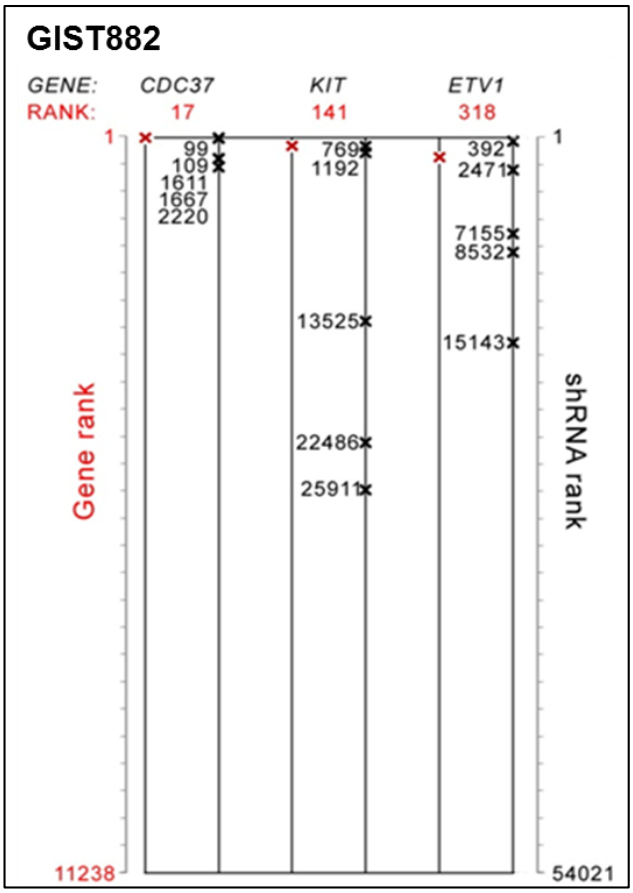


Figure 7: Most of the top 0.5% essential genes for GIST882 and GIST-T1 were commonly essential genes, based on their ranks in at least 8 of 12 non-GIST cancer cell lines of various lineages. However, six genes, including *CDC37*, were selectively essential in GIST882 and GIST-T1 compared with the non-GIST lines.

Table 1: Top 0.5% essential genes according to the second best scoring hairpin in GIST882 and GIST-T1 (n=56 for each). Genes in bold font (upper fields) scored top 0.5% in the gene distribution in GIST lines but not in 12 non-GIST reference cancer cell lines of various lineages (described by Luo et al.15). Genes in regular font (lower fields) scored top 0.5% in both GIST and the 12 non-GIST reference set.



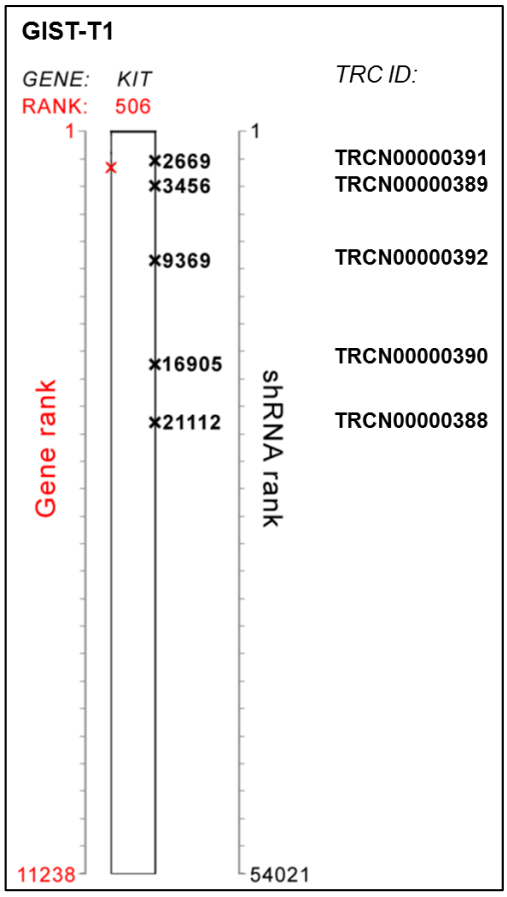


Figure 8: Gene ranks (red) and shRNA ranks (black) corresponding to *CDC37*, *KIT* and *ETV1* in GIST882 cells (left) and to *KIT* in GIST-T1 cells (right). Essential genes (oncogenes) rank on the top of the distribution

of HSP90 client proteins, including several kinases [Vaughan *et al.*, 2008; Smith and Workman, 2009], by mechanisms involving CDC37 homodimerization, CDC37-HSP90 heterodimerization and the formation of CDC37-kinase-HSP90 complexes [Xu *et al.*, 2012]. These observations suggest that CDC37 targeting might be a selective approach to derailing HSP90-mediated KIT oncoprotein chaperoning in GIST. The *KIT* oncogenic driver and the GIST-lineage-related transcription factor *ETV1* also scored as essential genes in these primary screens and serve as positive controls (**Figure 8**). In GIST-T1 cells, only one out of the five shRNAs targeting *KIT* was highly depleted, so *KIT* did not rank highly in the essential genes list; however, subsequent experiments showed that only the strongly depleted shRNA was highly effective at suppressing *KIT* in these cells (~70% knockdown) whereas the other four shRNAs produced <30% *KIT* knockdown (**Figures 8 and 9**).

Figure 9: Protein immunoblotting demonstrating low knockdown efficiency of the 5 KIT-targeting shRNAs present in the library, in GIST-T1 cells.

CDC37 interacts with KIT in GIST, maintaining KIT expression and cell survival

CDC37 expression was demonstrated by immunoblotting in the GIST cell lines (**Figure 10**). Interaction between CDC37 and KIT in these GISTs was demonstrated by co-immunoprecipitations using KIT and CDC37 antibodies in KIT-positive GIST882 and GIST-T1 cells, with KIT-negative GIST48B cells serving as a negative control (**Figure 10**). CDC37 shRNA-mediated knockdowns resulted in >90% reduction of KIT expression and activation in the KIT-dependent GIST882, GIST430 and GIST-T1 lines (**Figure 11**). KIT inhibition was associated with inactivation of downstream growth and survival signaling intermediates, including AKT. By contrast, AKT was not inhibited by CDC37 knockdown in the KIT-negative cell line GIST48B, suggesting that the observed inhibition of downstream signaling pathways in GIST882, GIST430 and GIST-T1 was KIT dependent (**Figure 11**).

Figure 10: CDC37 expression and CDC37-KIT co-immunoprecipitations in GIST cell lines, demonstrating CDC37:KIT interaction.

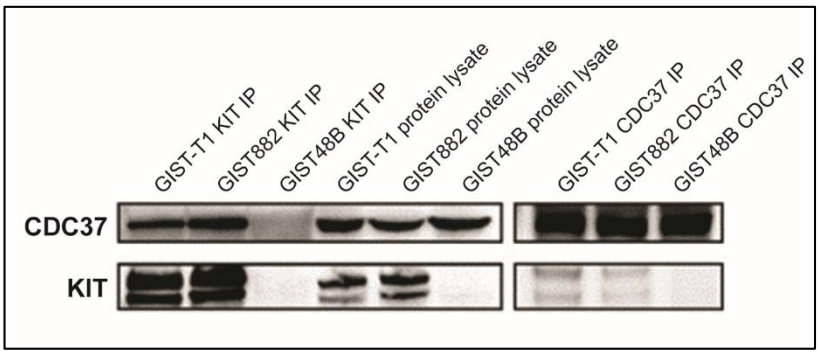
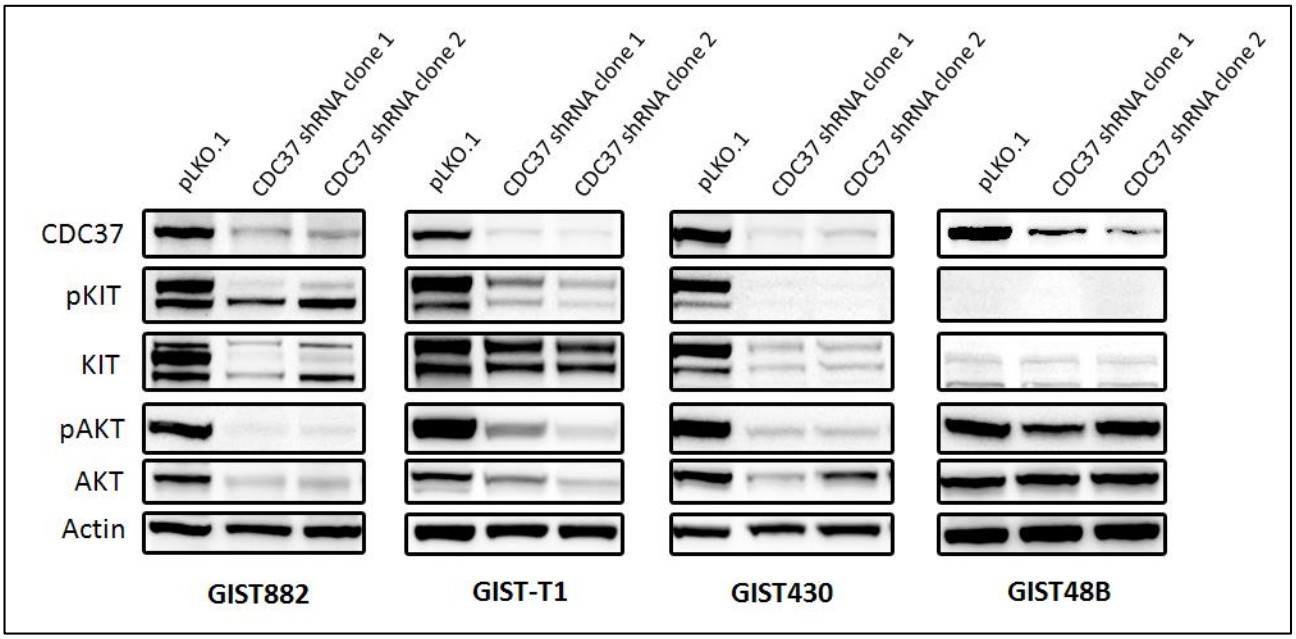


Figure 11: Persistent inhibition of KIT oncoprotein expression and phosphorylation, and decreased downstream signaling pathway activation in KITdependent GIST cells on shRNA-mediated CDC37 knockdown.



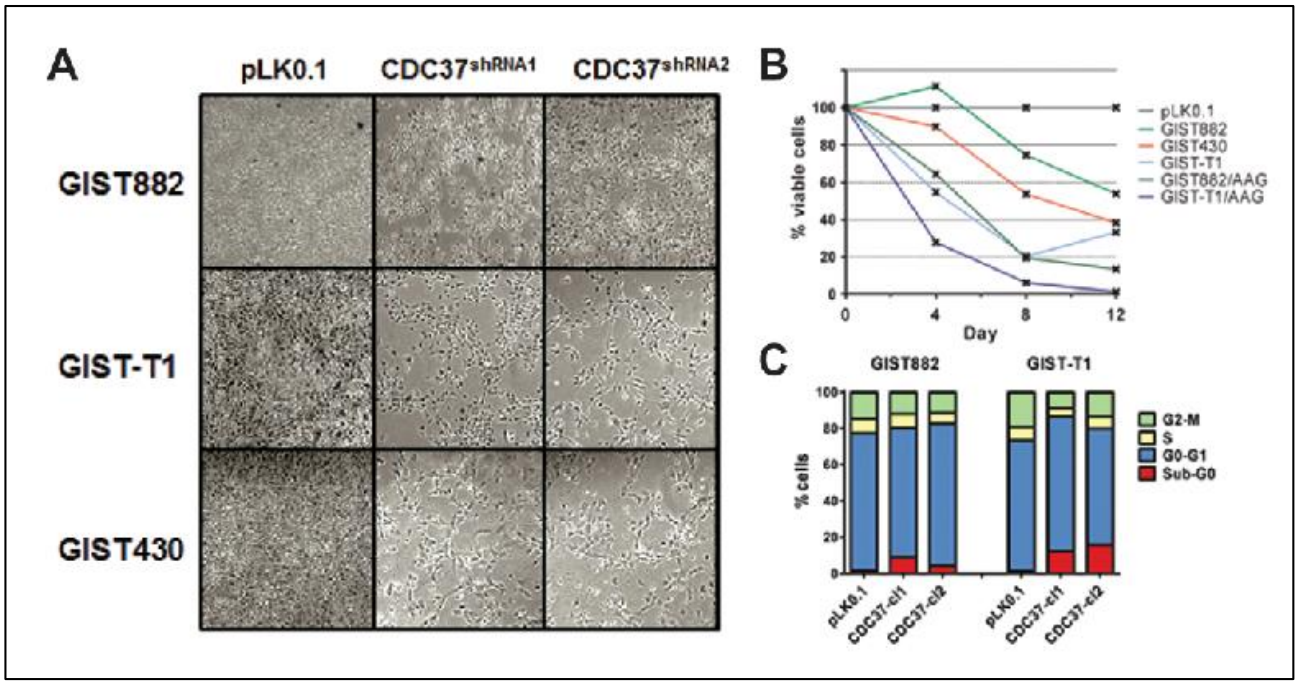


Figure 12: shRNA-mediated CDC37 knockdown inhibits viability of GISTs, including those resistant to imatinib (GIST430) and to the ansamycin-type HSP90-inhibitor 17-AAG (GIST882/ AAG and GIST-T1/AAG) as demons-trated by cell morphology **(A)**, ATP-based cell viability **(B)** and by the increase in the number of cells in sub-G0 phase of the cell cycle, 10 days after lentiviral infection and puromycin selection **(C)**.

CDC37 knockdown, unlike direct HSP90 inhibition [Chandarlapaty *et al.*, 2008], resulted in persistent inhibition of KIT expression for >20 days, indicating that GIST cells have few compensatory pathways for CDC37 function. The above-mentioned biochemical responses to CDC37 knockdown were accompanied by decreased GIST proliferation and survival as assessed by bright field microscopy and CellTiter-Glo assays (**Figures 12A and 12B**). Notably, these responses were also seen in GIST lines resistant to imatinib (GIST430) or to the ansamycin HSP90 inhibitor 17-AAG (GIST-T1/AAG and GIST882/AAG). CDC37 knockdown induced an increased sub-G0 cell cycle peak, consistent with a pro-apoptotic effect (**Figure 12C**).

shRNA-mediated CDC37 knockdown in GIST-T1 cells resulted in significantly decreased growth *in vivo*, with decreased tumor volume over a 6 week period, overall decreased tumor cellularity and decreased mitotic activity in subcutaneous mouse xenografts (**Figure 13**).

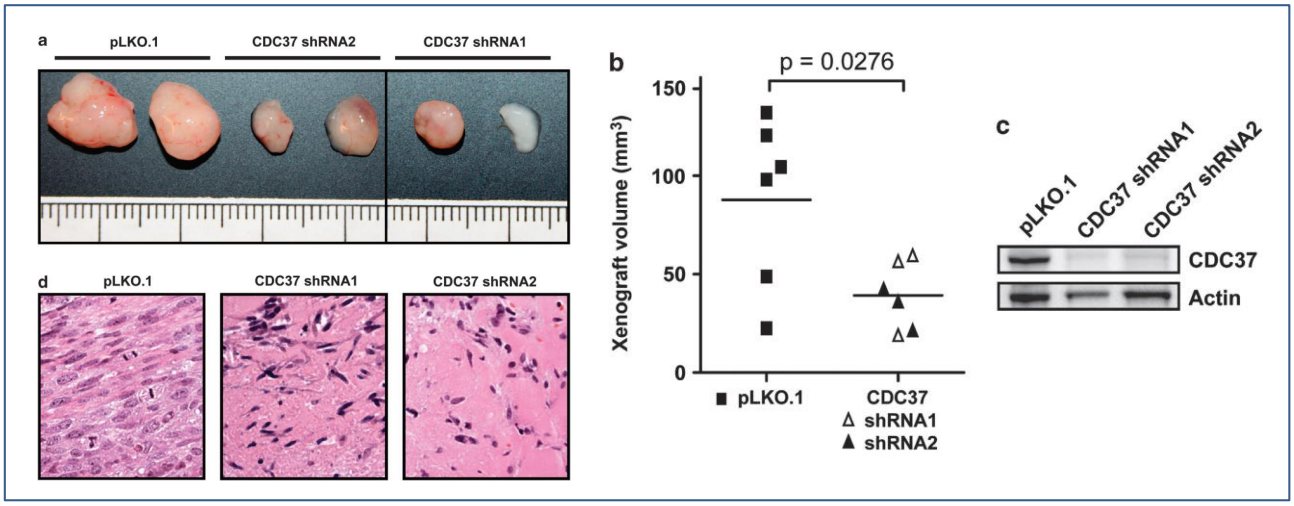
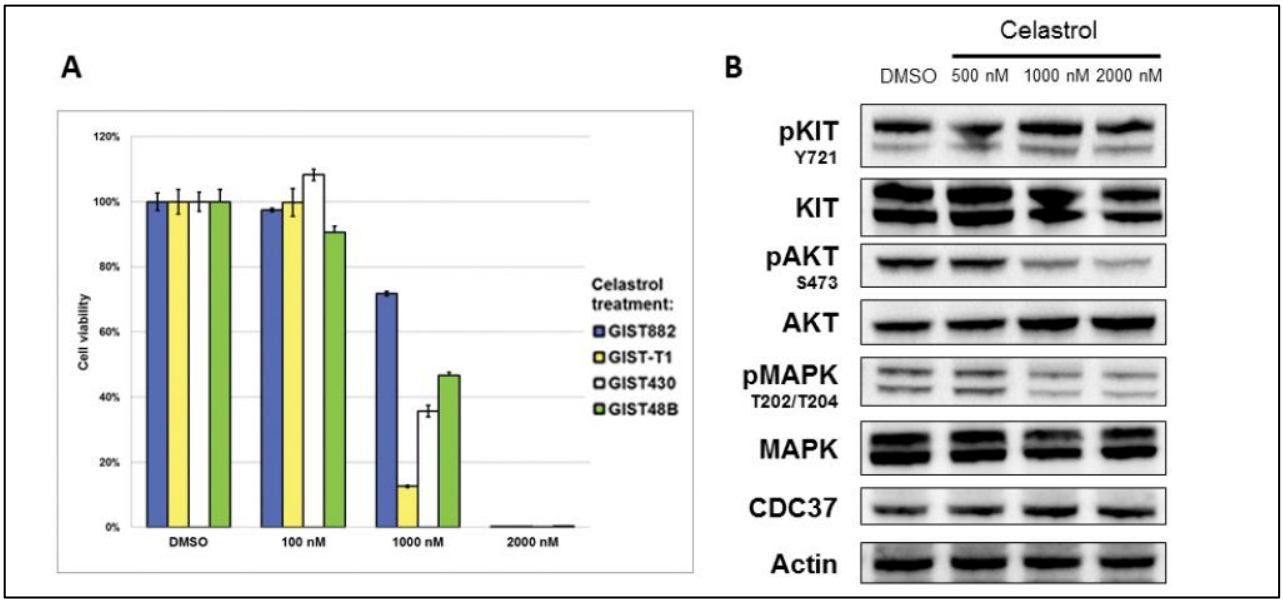


Figure 13: shRNA-mediated CDC37 knockdown inhibits growth of GIST xenografts in mice. **(a, b)** Significantly decreased tumor growth on CDC37 knockdown. **(c)** Western blot confirming inhibition of CDC37 expression in GIST-T1 cells infected with CDC37 shRNA1 and shRNA2, compared with pLKO.1 lentiviral vector. **(d)** Histologic evaluation of demonstrates sparsely cellular areas with no mitotic activity in CDC37-knockdown GIST xenografts, in comparison with highly cellular and mitotically active xenografts of pLKO.1-infected cells.

Celastrol does not enable selective CDC37:HSP90 pharmacologic inhibition

Preclinical pharmacologic validations were attempted using celastrol, a natural product reported to inhibit the HSP90:CDC37 interface [Zhang *et al.*, 2009]. Celastrol nonselectively inhibited viability of both KIT-dependent and KIT-independent (GIST48B) GIST cell lines (**Figure 14A**), and celastrol treatment did not result in dramatic dose-dependent inhibition of KIT expression or KIT activation, despite reduced AKT and mitogen-activated protein kinase phosphorylation (**Figure 14B**). These findings underscore the protean effects of celastrol, which are reported to

Figure 14: (A) Dose-dependent reduction of GIST cell viability by the HSP90:CDC37 interface inhibitor celastrol, despite persistent expression and activation of KIT **(B)**.



include proteasome inhibition [Zhang *et al.*, 2009], and which will likely preclude achieving CDC37:HSP90-inhibitory drug concentrations in the clinical setting. We therefore expect that effective clinical translation of CDC37 inhibition will require development of potent and more selective CDC37 inhibitors. Opportunities for selective CDC37 targeting might result from pharmacologic dysregulation of phosphorylation at CDC37 Ser13. This phosphorylation event—mediated by casein kinase 2—is requisite for CDC37 recruitment of kinase clients to the HSP90 complex, whereas CDC37 Ser13 dephosphorylation—mediated by protein phosphatase 5—is then necessary for priming CDC37 for tyrosine phosphorylation by YES1, ultimately enabling CDC37 and client release from the complex [Xu *et al.*, 2012] . Therefore, strategies targeting casein kinase 2, protein phosphatase 5 or YES1 might inhibit KIT-directed HSP90 functions in GIST.

In summary, we performed unbiased genome-wide loss-of-function genomic screens, which identified CDC37 as a compelling therapeutic target in GIST. CDC37 interacts with KIT oncoproteins in GIST and is an essential enabler of KIT oncogenic function. Preclinical validations highlight the selectivity and efficacy of targeting KIT through CDC37 in GIST, with advantages compared with direct HSP90 targeting including persistent downregulation of KIT expression and selectivity for only a subset of HSP90 client proteins. These observations suggest that selective targeting of CDC37 might be effective and less toxic than chronic HSP90 inhibition in GIST patients.

Discussion

Remarkable progress has been made in the past two decades towards understanding the biology of GIST, resulting in improved clinical outcomes through the rational development of targeted therapies. The discovery of KIT/PDGFRA activating mutations in GIST, followed by the extremely successful repurposing of imatinib for GIST treatment -from its original conception as a BCR-ABL inhibitor, for the treatment of chronic myelogenous leukemia-, has transformed the field of solid tumor therapeutics. More recently, the understanding of resistance mechanisms led to the approval of first sunitinib, and then regorafenib, as sequential second and third line treatments for progressing disease. At present, GIST represents the most successful example of translational research applied to the development of targeted therapies for solid tumors, having three targeted agents providing clinical benefit in sequential administration. Up to 10% of patients with metastatic disease achieve long term disease control (>10 years) on single-agent imatinib. Sunitinib and regorafenib provide further disease control of relatively short duration, and no alternative drugs have been demonstrated to provide additional clinical benefit. Ultimately, 90% of GIST patients relapse, and eventually succumb, to progressing GIST. There is a still an unmet clinical need, and hence the search for more effective inhibitors and novel therapeutic targets continues. In this context, the work presented herein aimed to identify biological dependencies in GIST cells that could reveal vulnerabilities to be exploited for therapeutic purposes. The identification of CDC37 as an essential protein for GIST cells represents such a dependency, and emphasizes the importance of the HSP90 chaperoning complex function for GIST biology.

Inhibition of HSP90 in GIST, and other RTK-driven tumors, has been an area of active investigation over the last decade [Workman, 2004]. Preclinical evidence supporting this therapeutic approach is very strong: the activated form of KIT/PDGFRA, the oncogenic driver of GIST cells, is structurally unstable and highly dependent on a proficient chaperoning machinery to function properly. Inhibition of the HSP90 complex results in decreased KIT phosphorylation, loss of activated signaling intermediates, including AKT an MAPK, and severely reduces GIST cell viability and proliferation [Bauer *et al.*, 2006]. Despite strong preclinical data, early clinical experience has been somewhat disappointing. A phase I clinical trial of retaspimycin hydrochloride (IPI-504), a geldanamycin-derivative HSP90 inhibitor, demonstrated activity in patients with advanced GIST who had progressed after treatment with imatinib and/or sunitinib, with a progression-free survival of 12 weeks and an overall response rate of 3% [Wagner *et al.*, 2013]. A larger scale phase III placebo-controlled international trial was terminated early, however, due to safety concerns of hepatic toxicity in the IPI-504 arm, highlighting the challenges of moving from a small phase I experience to broader

studies, even in the same patient population [Demetri *et al.*, 2010]. The apparent failure of HSP90 inhibition in this early trials can be attributed to intrinsic properties of the compounds used (geldanamycin derivatives), to the fact that the patients enrolled were heavily pre-treated, and to the drug administration schedule and dosing. Trials of alternative compounds, such as ganetespib (STA-9090), were designed to interrogate the duration of on-target activity in addition to safety profiles, and demonstrated that once-weekly dosing may be insufficient for optimal inhibition of KIT [Demetri *et al.*, 2010]. Improvements in drug scheduling and dosing, as well as new generation compounds are expected to lead to clinical benefit. The results of the experiments presented herein, in which the HSP90 co-chaperone CDC37 scored as one of the most essential genes in an unbiased genome-wide screen, highlight how the HSP90 machinery is one of the most critical nodes of GIST cells biology and justify the sustained efforts to improve therapeutic development against HSP90.

CDC37 targeting could provide some therapeutic advantages over direct HSP90 targeting. Recent estimates indicate that HSP90 inhibition alters the expression of several hundreds of cytosolic proteins [Samant *et al.*, 2012; Sharma *et al.*, 2012]. In contrast, CDC37 binds to just a fraction of those, by virtue of its kinase specificity [Vaughan *et al.*, 2008; Smith and Workman, 2009; Smith *et al.*, 2009]. This fact alone is likely to confer more specificity to CDC37 inhibition, and the fact that kinases are the main class of proteins disrupted provides selectivity of action on cancer cells, potentially increasing the therapeutic window. An important challenge for CDC37 targeting is the lack of compounds with clinical-grade pharmacologic activity. The natural product *celastrol* -derived from a plant known as Thunder of God Vine, widely utilized in traditional medicine to treat a variety of disorders- had been initially characterized to inhibit the HSP90:CDC37 interaction *in vitro* [Zhang *et al.*, 2009], but a series of additional publications describing a range of seemingly unrelated effects [Liu *et al.*, 2015] indicate that this compound lacks specificity, and probably acts upon multiple biochemical substrates in the cell. Our own results support this interpretation, since the decrease of GIST cell viability induced by celastrol was independent of the presence of KIT (**Figure 14**). Inhibiting the interaction between CDC37 and HSP90 would theoretically interfere with the function of the HSP90 complex [Taipale *et al.*, 2012], and several protein:protein interaction inhibitors have been hypothesized, but the relevance of each interactor within the complex is still poorly understood. Furthermore, direct interaction between HSP90 and CDC37 may not be necessary for proper function of the complex, and other components of the complex may be able to compensate for the lack of them [Smith *et al.*, 2015]. Modulation of CD37 activation, which occurs by phosphorylation of serine 13, may provide an additional point of pharmacologic attack on CDC37. The main regulators of phosphorylation at this site are CK2 and PPP5, which may be inhibited therapeutically. However, these kinase and phosphatase act on a large number of substrates, so their inhibition may lead to

undesired effects on other targets. We are actively evaluating these additional targets in the laboratory, and we will follow up on the most promising leads that could provide a therapeutic effect.

There are biological and technical aspects regarding the use of shRNA libraries that deserve consideration. First, the level of interference of a shRNA on the target transcript is variable, depending on the design of the hairpin, the specific sequence, and the transcriptional and cellular context. As a result, the *on-target* knockdown efficiency of any given shRNA is variable, and to some extent unpredictable. Second, in addition to knocking down the intended target gene transcript by complementarity of its 21 nucleotide sequence, shRNAs knock down transcripts of other genes and produce *off-target* effects. Most of these are the result of the shRNAs behaving like miRNAs, by virtue of a shared sequence of 6-8 nucleotides (comprised within nucleotides 2-8), known as the seed region [Doench *et al.*, 2003]. These seed regions allow the hairpins to bind the transcripts of unintended groups of genes and reduce their availability via the miRNA pathway [Jackson *et al.*, 2006], and have been proven to significantly contribute to the phenotype induced by the shRNAs in several cell contexts [Franceschini *et al.*, 2014; Singh *et al.*, 2015]. For these reasons, redundancy is introduced into shRNA libraries, and each gene is targeted by multiple shRNAs. In the case of the TRC library utilized here, there are 6 shRNAs per gene on average. The most recently developed 'ultracomplex' libraries include 25-50 shRNAs per gene and thousands of negative control sequences, adding up to over 500,000 shRNAs [Kampmann *et al.*, 2015]. Computational approaches allow to identify the signal produced by on target effects, and subtract or at least quantify the magnitude of off-target effects driven by seed sequences and interpret the data in this context [Sigoillot *et al.*, 2012; Zhong *et al.*, 2014]. Occasionally, these analyses help identify biological events driven by microRNAs [Schultz *et al.*, 2011; Buehler *et al.*, 2012]. For the results presented here, we performed a customized analysis with a biocomputational tool developed at the Broad Institute to identify seed effects in the data (this tool is designated miRkat, and will be made available to the community in upcoming months within the DEMETER algorithm, which estimates the relative contributions of hairpin-related and seed-related effects for each gene); off-target effects in our datasets were not statistically significant, and did not mask the signal produced by on-target effects.

These biological and technical features of shRNAs influence the computational analyses performed to collapse shRNA-level information into gene-level information for large scale shRNA screens. The simplest approach, a rank of hairpins according to their relative enrichment and depletion at the end of the experiment, is prone to false positive results by over-representation of the hairpins with more severe off-target effects. To enrich for on-target effects, there are statistical approaches that consider the ranks of all the shRNAs targeting each gene, to generate an *RNAi Gene Enrichment*

Ranking (RIGER). Using a non-parametric method based on Kolmogorov-Smirnov statistics, it is possible to assign a score to each gene based on the depletion or enrichment of the corresponding shRNA set within the absolute rank, based on the comparison of two classes. This method is similar to the widely used gene set enrichment analysis (GSEA) and has been described and extensively validated in the literature [Luo *et al.*, 2008; Barbie *et al.*, 2009]. Empirically, it has been observed that ranking the genes according to the second-best-scoring hairpin in the absolute distribution provides a very accurate rank, which captures useful biological information in large scale genomic shRNA screens. This "second-best" method provides a simple mechanism to neutralize outliers, and has been applied successfully to screen multiple different biological systems [Cheung *et al.*, 2011; Marcotte *et al.*, 2012; Zhang *et al.*, 2013]. We analyzed our datasets with both RIGER and the "second best" method, and the results at the extremes of the rank were very similar, indicating a high signal-to-noise ratio. As the number of screens with similar platforms increases, it is becoming possible to analyze the performance of each reagent (shRNA) across multiple experiments, and assign them a "quality score" that can be used to weight the results and generate more informative ranks and hit lists. Novel algorithms such as ATARiS and DEMETER take into consideration the results of multiple experiments and provide information about the quality and performance of each reagent, allowing to extract more information from datasets with more noise [Shao *et al.*, 2013].

Beyond these technical and biological considerations of the screens themselves, the most important analyses are those relative to the biology of the model of study. The systematic application of functional genomics has the potential to reveal the basic blueprint of cellular survival and proliferation. Moreover, these approaches can uncover genes that are exclusively essential for cancer cells, genes that mediate the cellular response to therapy, or those that behave as synthetic lethals with a given intervention, all of which are potentially useful for the development of specific anticancer therapies. Cumulative experience in the field has revealed several challenges inherent to functional genomic technologies, most of them related to off-target effects and low signal-to-noise ratio in some systems [Hart *et al.*, 2014]. Ongoing efforts aim to systematically collect and compare the numerous datasets generated by functional genomic experiments, to build gold-standard databases and reference sets to provide a working framework that will maximize the output of each individual experiment [Hart *et al.*, 2014]. Triangulation of functional data with structural genomic data and chemical screens will maximize the yield of all these approaches collectively [Jerby-Arnon *et al.*, 2014].

Important questions remain to be answered regarding GIST biology: the process of tumor initiation, oncogenic progression, the degree and clinical significance of tumor heterogeneity, histopathological correlation with molecular characteristics, the biology of rare histological subtypes, and precise prognostication, to name a few, all can provide important clues to further improvement patient management. Our laboratory is actively working on all these questions, utilizing state-of-the-art technologies and experimental approaches that are generating an unprecedented wealth of data. It is not an overstatement to affirm that our understanding of GIST biology, tumor heterogeneity, and mechanisms of drug resistance is improving every week. It is fair to expect that in the next few years there will be substantial advances in the treatment of GIST, which will maximize long-term disease control and hopefully improve GIST patients' outcomes.

Conclusiones

1. En el marco de este trabajo hemos desarrollado con éxito una plataforma de genómica funcional para el estudio genético sistemático de modelos celulares de sarcoma.

2. Con esta plataforma genómica es posible analizar individualmente la función de más de 12,000 genes, utilizando librerías de RNA interferente que permiten silenciar cada gen de manera específica, en un contexto celular fisiológico.

3. Aplicando esta tecnología funcional de alto rendimiento hemos realizado experimentos de pérdida de función a escala genómica en células de tumor del estroma gastrointestinal (GIST).

4. La proteína CDC37 es una nueva diana terapéutica en GIST, esencial para la biología tumoral, identificada mediante experimentos de screening a escala genómica.

5. CDC37 interacciona con la oncoproteína KIT en las células de GIST, y es un factor indispensable para la función oncogénica de KIT.

6. Experimentos de validación preclínica demuestran que la inhibición de KIT a través de CDC37 puede realizarse de forma selectiva y eficaz en GIST.

7. La inhibición de KIT mediante CDC37 presenta ventajas en comparación con la inhibición de HSP90, como son la inhibición prolongada de la expresión de KIT y la reducción de la población de proteínas afectadas a solo una fracción de las proteínas “cliente” de HSP90.

8. La inhibición selectiva de CDC37 es una aproximación terapéutica efectiva y menos tóxica que la inhibición de HSP90 en pacientes con GIST.

Bibliografía

Agaimy A, Wunsch PH, Dirnhofer S, Bihl MP, Terracciano LM, Tornillo L. Microscopic gastrointestinal stromal tumors in esophageal and intestinal surgical resection specimens: A clinicopathologic, immunohistochemical, and molecular study of 19 lesions. *Am J Surg Pathol* 2008; **32**:867-73.

Agaimy A, Wunsch PH, Hofstaedter F, Blaszyk H, Rummele P, Gaumann A, Dietmaier W, Hartmann A. Minute gastric sclerosing stromal tumors (GIST tumorlets) are common in adults and frequently show c-KIT mutations. *Am J Surg Pathol* 2007; **31**:113-20.

Antonescu CR, Romeo S, Zhang L, Nafa K, Hornick JL, Nielsen GP, Mino-Kenudson M, Huang HY, Mosquera JM, Dei Tos PA, Fletcher CD. Dedifferentiation in gastrointestinal stromal tumor to an anaplastic KIT-negative phenotype: A diagnostic pitfall: Morphologic and molecular characterization of 8 cases occurring either de novo or after imatinib therapy. *Am J Surg Pathol* 2013; **37**:385-92.

Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, Schinzel AC, Sandy P, Meylan E, Scholl C, Frohling S, Chan EM, Sos ML, Michel K, Mermel C, Silver SJ, Weir BA, Reiling JH, Sheng Q, Gupta PB, Wadlow RC, Le H, Hoersch S, Wittner BS, Ramaswamy S, Livingston DM, Sabatini DM, Meyerson M, Thomas RK, Lander ES, Mesirov JP, Root DE, Gilliland DG, Jacks T, Hahn WC. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* 2009; **462**:108-12.

Bauer S, Duensing A, Demetri GD, Fletcher JA. KIT oncogenic signaling mechanisms in imatinib-resistant gastrointestinal stromal tumor: PI3-kinase/AKT is a crucial survival pathway. *Oncogene* 2007; **26**:7560-8.

Bauer S, Yu LK, Demetri GD, Fletcher JA. Heat shock protein 90 inhibition in imatinib-resistant gastrointestinal stromal tumor. *Cancer Res* 2006; **66**:9153-61.

Blanke CD, Demetri GD, von MM, Heinrich MC, Eisenberg B, Fletcher JA, Corless CL, Fletcher CD, Roberts PJ, Heinz D, Wehre E, Nikolova Z, Joensuu H. Long-term results from a randomized phase II trial of standard- versus higher-dose imatinib mesylate for patients with unresectable or metastatic gastrointestinal stromal tumors expressing KIT. *J Clin Oncol* 2008; **26**:620-5.

Boehm JS, Hahn WC. Towards systematic functional characterization of cancer genomes. *Nat Rev Genet* 2011; **12**:487-98.

Buehler E, Khan AA, Marine S, Rajaram M, Bahl A, Burchard J, Ferrer M. siRNA off-target effects in genome-wide screens identify signaling pathway members. *Sci Rep* 2012; **2**:428.

Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; **455**:1061-8.

Carney JA, Stratakis CA. Familial paraganglioma and gastric stromal sarcoma: A new syndrome distinct from the carney triad. *Am J Med Genet* 2002; **108**:132-9.

Chandarlapaty S, Sawai A, Ye Q, Scott A, Silinski M, Huang K, Fadden P, Partdrige J, Hall S, Steed P, Norton L, Rosen N, Solit DB. SNX2112, a synthetic heat shock protein 90 inhibitor, has potent antitumor activity against HER kinase-dependent cancers. *Clin Cancer Res* 2008; **14**:240-8.

Chen Y, Tzeng CC, Liou CP, Chang MY, Li CF, Lin CN. Biological significance of chromosomal imbalance aberrations in gastrointestinal stromal tumors. *J Biomed Sci* 2004; **11**:65-71.

Cheung HW, Cowley GS, Weir BA, Boehm JS, Rusin S, Scott JA, East A, Ali LD, Lizotte PH, Wong TC, Jiang G, Hsiao J, Mermel CH, Getz G, Barretina J, Gopal S, Tamayo P, Gould J, Tsherniak A, Stransky N, Luo B, Ren Y, Drapkin R, Bhatia SN, Mesirov JP, Garraway LA, Meyerson M, Lander ES, Root DE, Hahn WC. Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. *Proc Natl Acad Sci U S A* 2011; **108**:12372-7.

Chi P, Chen Y, Zhang L, Guo X, Wongvipat J, Shamu T, Fletcher JA, Dewell S, Maki RG, Zheng D, Antonescu CR, Allis CD, Sawyers CL. ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. *Nature* 2010; **467**:849-53.

Corless CL, Barnett CM, Heinrich MC. Gastrointestinal stromal tumours: Origin and molecular oncology. *Nat Rev Cancer* 2011; **11**:865-78.

Corless CL, Fletcher JA, Heinrich MC. Biology of gastrointestinal stromal tumors. *J Clin Oncol* 2004; **22**:3813-25.

Corless CL, Schroeder A, Griffith D, Town A, McGreevey L, Harrell P, Shiraga S, Bainbridge T, Morich J, Heinrich MC. PDGFRA mutations in gastrointestinal stromal tumors: Frequency, spectrum and in vitro sensitivity to imatinib. *J Clin Oncol* 2005; **23**:5357-64.

Cowley GS, Weir BA, Vazquez F, Tamayo P, Scott JA, Rusin S, East-Seletsky A, Ali LD, Gerath WF, Pantel SE, Lizotte PH, Jiang G, Hsiao J, Tsherniak A, Dwinell E, Aoyama S, Okamoto M, Harrington W, Gelfand E, Green TM, Tomko MJ, Gopal S, Wong TC, Li H, Howell S, Stransky N, Liefeld T, Jang D, Bistline J, Hill Meyers B, Armstrong SA, Anderson KC, Stegmaier K, Reich M, Pellman D, Boehm JS, Mesirov JP, Golub TR, Root DE, Hahn WC. Parallel genome-scale loss of function screens in 216 cancer cell lines for the identification of context-specific genetic dependencies. *Sci Data* 2014; **1**:140035.

Debiec-Rychter M, Cools J, Dumez H, Sciot R, Stul M, Mentens N, Vranckx H, Wasag B, Prenen H, Roesel J, Hagemeijer A, Van OA, Marynen P. Mechanisms of resistance to imatinib mesylate in gastrointestinal stromal tumors and activity of the PKC412 inhibitor against imatinib-resistant mutants. *Gastroenterology* 2005; **128**:270-9.

Dematteo RP, Ballman KV, Antonescu CR, Maki RG, Pisters PW, Demetri GD, Blackstein ME, Blanke CD, von Mehren M, Brennan MF, Patel S, McCarter MD, Polikoff JA, Tan BR, Owzar K, American College of Surgeons Oncology Group (ACOSOG) Intergroup Adjuvant GIST Study Team. Adjuvant imatinib mesylate after resection of localised, primary gastrointestinal stromal tumour: A randomised, double-blind, placebo-controlled trial. *Lancet* 2009; **373**:1097-104.

Dematteo RP, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF. Two hundred gastrointestinal stromal tumors: Recurrence patterns and prognostic factors for survival. *Ann Surg* 2000; **231**:51-8.

Demetri GD, Benjamin RS, Blanke CD, Blay JY, Casali P, Choi H, Corless CL, Debiec-Rychter M, Dematteo RP, Ettinger DS, Fisher GA, Fletcher CD, Gronchi A, Hohenberger P, Hughes M, Joensuu H, Judson I, Le CA, Maki RG, Morse M, Pappo AS, Pisters PW, Raut CP, Reichardt P, Tyler DS, Van den Abbeele AD, von MM, Wayne JD, Zalcberg J. NCCN task force report: Management of

patients with gastrointestinal stromal tumor (GIST)--update of the NCCN clinical practice guidelines. *J Natl Compr Canc Netw* 2007; **5 Suppl 2**:S1-29.

Demetri GD, Le Cesne A, Von Mehren M, Chmielowski B, Bauer S, Chow WA, *et al.* Final results from a phase III study of IPI-504 (retaspimycin hydrochloride) versus placebo in patients with GIST following failure of kinase inhibitor therapies. [abstract]. 2010 In: Proceedings of the Gastrointestinal Cancers Symposium; 2010 Jan 22. Alexandria, VA: American Society of Clinical Oncology.

Demetri GD, Reichardt P, Kang YK, Blay JY, Rutkowski P, Gelderblom H, Hohenberger P, Leahy M, von Mehren M, Joensuu H, Badalamenti G, Blackstein M, Le Cesne A, Schoffski P, Maki RG, Bauer S, Nguyen BB, Xu J, Nishida T, Chung J, Kappeler C, Kuss I, Laurent D, Casali PG, GRID study investigators. Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): An international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 2013; **381**:295-302.

Demetri GD, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, Verweij J, McArthur G, Judson IR, Heinrich MC, Morgan JA, Desai J, Fletcher CD, George S, Bello CL, Huang X, Baum CM, Casali PG. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: A randomised controlled trial. *Lancet* 2006; **368**:1329-38.

Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002; **347**:472-80.

Desai J, Shankar S, Heinrich MC, Fletcher JA, Fletcher CD, Manola J, Morgan JA, Corless CL, George S, Tuncali K, Silverman SG, Van den Abbeele AD, van Sonnenberg E, Demetri GD. Clonal evolution of resistance to imatinib in patients with metastatic gastrointestinal stromal tumors. *Clin Cancer Res* 2007; **13**:5398-405.

Doench JG, Petersen CP, Sharp PA. siRNAs can function as miRNAs. *Genes Dev* 2003; **17**:438-42.

Doyle LA, Nelson D, Heinrich MC, Corless CL, Hornick JL. Loss of succinate dehydrogenase subunit B (SDHB) expression is limited to a distinctive subset of gastric wild-type gastrointestinal stromal tumours: A comprehensive genotype-phenotype correlation study. *Histopathology* 2012; **61**:801-9.

Duensing A, Joseph NE, Medeiros F, Smith F, Hornick JL, Heinrich MC, Corless CL, Demetri GD, Fletcher CD, Fletcher JA. Protein kinase C theta (PKCtheta) expression and constitutive activation in gastrointestinal stromal tumors (GISTs). *Cancer Res* 2004; **64**:5127-31.

Duensing A, Medeiros F, McConarty B, Joseph NE, Panigrahy D, Singer S, Fletcher CD, Demetri GD, Fletcher JA. Mechanisms of oncogenic KIT signal transduction in primary gastrointestinal stromal tumors (GISTs). *Oncogene* 2004; **23**:3999-4006.

Edris B, Willingham SB, Weiskopf K, Volkmer AK, Volkmer JP, Muhlenberg T, Montgomery KD, Contreras-Trujillo H, Czechowicz A, Fletcher JA, West RB, Weissman IL, van de Rijn M. Anti-KIT monoclonal antibody inhibits imatinib-resistant gastrointestinal stromal tumor growth. *Proc Natl Acad Sci U S A* 2013; **110**:3501-6.

El-Rifai W, Sarlomo-Rikala M, Andersson LC, Knuutila S, Miettinen M. DNA sequence copy number changes in gastrointestinal stromal tumors: Tumor progression and prognostic significance. *Cancer Res* 2000; **60**:3899-903.

Falchook GS, Trent JC, Heinrich MC, Beadling C, Patterson J, Bastida CC, Blackman SC, Kurzrock R. BRAF mutant gastrointestinal stromal tumor: First report of regression with BRAF inhibitor dabrafenib (GSK2118436) and whole exomic sequencing for analysis of acquired resistance. *Oncotarget* 2013; **4**:310-5.

Fletcher JA, Corless C, Dimitrijevic S. *Mechanisms of resistance to imatinib mesylate (IM) in advanced gastrointestinal stromal tumors (GIST).* Vol 22. 2003. 815 pp.

Fletcher CD, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach. *Hum Pathol* 2002; **33**:459-65.

Floris G, Wozniak A, Sciot R, Li H, Friedman L, Van Looy T, Wellens J, Vermaelen P, Deroose CM, Fletcher JA, Debiec-Rychter M, Schoffski P. A potent combination of the novel PI3K inhibitor, GDC-0941, with imatinib in gastrointestinal stromal tumor xenografts: Long-lasting responses after treatment withdrawal. *Clin Cancer Res* 2013; **19**:620-30.

Franceschini A, Meier R, Casanova A, Kreibich S, Daga N, Andritschke D, Dilling S, Ramo P, Emmenlauer M, Kaufmann A, Conde-Alvarez R, Low SH, Pelkmans L, Helenius A, Hardt WD, Dehio C, von Mering C. Specific inhibition of diverse pathogens in human cells by synthetic microRNA-like oligonucleotides inferred from RNAi screens. *Proc Natl Acad Sci U S A* 2014; **111**:4548-53.

Gasparotto D, Rossi S, Bearzi I, Doglioni C, Marzotto A, Hornick JL, Grizzo A, Sartor C, Mandolesi A, Sciot R, Debiec-Rychter M, Dei Tos AP, Maestro R. Multiple primary sporadic gastrointestinal stromal tumors in the adult: An underestimated entity. *Clin Cancer Res* 2008; **14**:5715-21.

George S, Wang Q, Heinrich MC, Corless CL, Zhu M, Butrynski JE, Morgan JA, Wagner AJ, Choy E, Tap WD, Yap JT, Van den Abbeele AD, Manola JB, Solomon SM, Fletcher JA, von Mehren M, Demetri GD. Efficacy and safety of regorafenib in patients with metastatic and/or unresectable GI stromal tumor after failure of imatinib and sunitinib: A multicenter phase II trial. *J Clin Oncol* 2012; **30**:2401-7.

Haller F, Lobke C, Ruschhaupt M, Cameron S, Schulten HJ, Schwager S, von HA, Gunawan B, Langer C, Ramadori G, Sultmann H, Poustka A, Korf U, Fuzesi L. Loss of 9p leads to p16INK4A down-regulation and enables RB/E2F1-dependent cell cycle promotion in gastrointestinal stromal tumours (GISTs). *J Pathol* 2008; **215**:253-62.

Hart T, Brown KR, Sircoulomb F, Rottapel R, Moffat J. Measuring error rates in genomic perturbation screens: Gold standards for human functional genomics. *Mol Syst Biol* 2014; **10**:733.

Heinrich MC, Fletcher JA, Anjum R, Serrano-Garcia C, Vodala S, Bauer S, Town A, Zhu M, Ning Y, Eilers G, Griffith D, Patterson J, McKinley A, Wang FY, Garner AP, Rivera VM. *Use of ponatinib to inhibit kinase mutations associated with drug-resistant gastrointestinal stromal tumors (GIST).* Vol 31 (suppl; abstr 10509). 2013.

Heinrich MC, Corless CL, Blanke CD, Demetri GD, Joensuu H, Roberts PJ, Eisenberg BL, von Mehren M, Fletcher CD, Sandau K, McDougall K, Ou WB, Chen CJ, Fletcher JA. Molecular correlates of imatinib resistance in gastrointestinal stromal tumors. *J Clin Oncol* 2006; **24**:4764-74.

Heinrich MC, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, McGreevey LS, Chen CJ, Van den Abbeele AD, Druker BJ, Kiese B, Eisenberg B, Roberts PJ, Singer S, Fletcher CD, Silberman S, Dimitrijevic S, Fletcher JA. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol* 2003; **21**:4342-9.

Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Haley A, Town A, Demetri GD, Fletcher CD, Fletcher JA. PDGFRA activating mutations in gastrointestinal stromal tumors. *Science* 2003; **299**:708-10.

Heinrich MC, Maki RG, Corless CL, Antonescu CR, Harlow A, Griffith D, Town A, McKinley A, Ou WB, Fletcher JA, Fletcher CD, Huang X, Cohen DP, Baum CM, Demetri GD. Primary and secondary kinase genotypes correlate with the biological and clinical activity of sunitinib in imatinib-resistant gastrointestinal stromal tumor. *J Clin Oncol* 2008; **26**:5352-9.

Heinrich MC, Owzar K, Corless CL, Hollis D, Borden EC, Fletcher CD, Ryan CW, von Mehren M, Blanke CD, Rankin C, Benjamin RS, Bramwell VH, Demetri GD, Bertagnolli MM, Fletcher JA. Correlation of kinase genotype and clinical outcome in the north american intergroup phase III trial of imatinib mesylate for treatment of advanced gastrointestinal stromal tumor: CALGB 150105 study by cancer and leukemia group B and southwest oncology group. *J Clin Oncol* 2008; **26**:5360-7.

Henze J, Muhlenberg T, Simon S, Grabellus F, Rubin B, Taeger G, Schuler M, Treckmann J, Debiec-Rychter M, Taguchi T, Fletcher JA, Bauer S. p53 modulation as a therapeutic strategy in gastrointestinal stromal tumors. *PLoS One* 2012; **7**:e37776.

Hornick JL, Fletcher CD. The role of KIT in the management of patients with gastrointestinal stromal tumors. *Hum Pathol* 2007; **38**:679-87.

Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS. Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *RNA* 2006; **12**:1179-87.

Janeway KA, Kim SY, Lodish M, Nose V, Rustin P, Gaal J, Dahia PL, Liegl B, Ball ER, Raygada M, Lai AH, Kelly L, Hornick JL, O'Sullivan M, de Krijger RR, Dinjens WN, Demetri GD, Antonescu CR, Fletcher JA, Helman L, Stratakis CA. Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. *Proc Natl Acad Sci U S A* 2011; **108**:314-8.

Jerby-Arnon L, Pfetzer N, Waldman YY, McGarry L, James D, Shanks E, Seashore-Ludlow B, Weinstock A, Geiger T, Clemons PA, Gottlieb E, Ruppin E. Predicting cancer-specific vulnerability via data-driven detection of synthetic lethality. *Cell* 2014; **158**:1199-209.

Joensuu H, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, Silberman S, Capdeville R, Dimitrijevic S, Druker B, Demetri GD. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N Engl J Med* 2001; **344**:1052-6.

Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, Emery CM, Stransky N, Cogdill AP, Barretina J, Caponigro G, Hieronymus H, Murray RR, Salehi-Ashtiani K, Hill DE, Vidal M, Zhao JJ, Yang X, Alkan O, Kim S, Harris JL, Wilson CJ, Myer VE, Finan PM, Root DE,

Roberts TM, Golub T, Flaherty KT, Dummer R, Weber BL, Sellers WR, Schlegel R, Wargo JA, Hahn WC, Garraway LA. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* 2010; **468**:968-72.

Kampmann M, Horlbeck MA, Chen Y, Tsai JC, Bassik MC, Gilbert LA, Villalta JE, Kwon SC, Chang H, Kim VN, Weissman JS. Next-generation libraries for robust RNA interference-based genome-wide screens. *Proc Natl Acad Sci U S A* 2015; **112**:E3384-91.

Kawanowa K, Sakuma Y, Sakurai S, Hishima T, Iwasaki Y, Saito K, Hosoya Y, Nakajima T, Funata N. High incidence of microscopic gastrointestinal stromal tumors in the stomach. *Hum Pathol* 2006; **37**:1527-35.

Khalili JS, Yu X, Wang J, Hayes BC, Davies MA, Lizee G, Esmaeli B, Woodman SE. Combination small molecule MEK and PI3K inhibition enhances uveal melanoma cell death in a mutant GNAQ- and GNA11-dependent manner. *Clin Cancer Res* 2012; **18**:4345-55.

Liegl B, Hornick JL, Antonescu CR, Corless CL, Fletcher CD. Rhabdomyosarcomatous differentiation in gastrointestinal stromal tumors after tyrosine kinase inhibitor therapy: A novel form of tumor progression. *Am J Surg Pathol* 2009; **33**:218-26.

Liegl B, Hornick JL, Corless CL, Fletcher CD. Monoclonal antibody DOG1.1 shows higher sensitivity than KIT in the diagnosis of gastrointestinal stromal tumors, including unusual subtypes. *Am J Surg Pathol* 2009; **33**:437-46.

Liegl B, Kepten I, Le C, Zhu M, Demetri GD, Heinrich MC, Fletcher CD, Corless CL, Fletcher JA. Heterogeneity of kinase inhibitor resistance mechanisms in GIST. *J Pathol* 2008; **216**:64-74.

Liegl-Atzwanger B, Fletcher JA, Fletcher CD. Gastrointestinal stromal tumors. *Virchows Arch* 2010; **456**:111-27.

Liu J, Lee J, Salazar Hernandez MA, Mazitschek R, Ozcan U. Treatment of obesity with celastrol. *Cell* 2015; **161**:999-1011.

Long KB, Butrynski JE, Blank SD, Ebrahim KS, Dressel DM, Heinrich MC, Corless CL, Hornick JL. Primary extragastrointestinal stromal tumor of the pleura: Report of a unique case with genetic confirmation. *Am J Surg Pathol* 2010; **34**:907-12.

Luo B, Cheung HW, Subramanian A, Sharifnia T, Okamoto M, Yang X, Hinkle G, Boehm JS, Beroukhim R, Weir BA, Mermel C, Barbie DA, Awad T, Zhou X, Nguyen T, Piqani B, Li C, Golub TR, Meyerson M, Hacohen N, Hahn WC, Lander ES, Sabatini DM, Root DE. Highly parallel identification of essential genes in cancer cells. *Proc Natl Acad Sci U S A* 2008; **105**:20380-5.

Marcotte R, Brown KR, Suarez F, Sayad A, Karamboulas K, Krzyzanowski PM, Sircoulomb F, Medrano M, Fedyshyn Y, Koh JL, van Dyk D, Fedyshyn B, Luhova M, Brito GC, Vizeacoumar FJ, Vizeacoumar FS, Datti A, Kasimer D, Buzina A, Mero P, Misquitta C, Normand J, Haider M, Ketela T, Wrana JL, Rottapel R, Neel BG, Moffat J. Essential gene profiles in breast, pancreatic, and ovarian cancer cells. *Cancer Discov* 2012; **2**:172-89.

Medeiros F, Corless CL, Duensing A, Hornick JL, Oliveira AM, Heinrich MC, Fletcher JA, Fletcher CD. KIT-negative gastrointestinal stromal tumors: Proof of concept and therapeutic implications. *Am J Surg Pathol* 2004; **28**:889-94.

Miettinen M, Lasota J. Gastrointestinal stromal tumors - definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. *Virchows Arch* 2001; **438**:1-12.

Miettinen M, Fetsch JF, Sobin LH, Lasota J. Gastrointestinal stromal tumors in patients with neurofibromatosis 1: A clinicopathologic and molecular genetic study of 45 cases. *Am J Surg Pathol* 2006; **30**:90-6.

Miettinen M, Lasota J. Gastrointestinal stromal tumors. *Gastroenterol Clin North Am* 2013; **42**:399-415.

Miettinen M, Lasota J. Gastrointestinal stromal tumors: Pathology and prognosis at different sites. *Semin Diagn Pathol* 2006; **23**:70-83.

Miettinen M, Monihan JM, Sarlomo-Rikala M, Kovatich AJ, Carr NJ, Emory TS, Sobin LH. Gastrointestinal stromal tumors/smooth muscle tumors (GISTs) primary in the omentum and mesentery: Clinicopathologic and immunohistochemical study of 26 cases. *Am J Surg Pathol* 1999; **23**:1109-18.

Miettinen M, Wang ZF, Lasota J. DOG1 antibody in the differential diagnosis of gastrointestinal stromal tumors: A study of 1840 cases. *Am J Surg Pathol* 2009; **33**:1401-8.

Nilsson B, Bumming P, Meis-Kindblom JM, Oden A, Dortok A, Gustavsson B, Sablinska K, Kindblom LG. Gastrointestinal stromal tumors: The incidence, prevalence, clinical course, and prognostication in the preimatinib mesylate era--a population-based study in western sweden. *Cancer* 2005; **103**:821-9.

Nishida T, Hirota S, Taniguchi M, Hashimoto K, Isozaki K, Nakamura H, Kanakura Y, Tanaka T, Takabayashi A, Matsuda H, Kitamura Y. Familial gastrointestinal stromal tumours with germline mutation of the KIT gene. *Nat Genet* 1998; **19**:323-4.

Novelli M, Rossi S, Rodriguez-Justo M, Taniere P, Seddon B, Toffolatti L, Sartor C, Hogendoorn PC, Sciot R, Van Glabbeke M, Verweij J, Blay JY, Hohenberger P, Flanagan A, Dei Tos AP. DOG1 and CD117 are the antibodies of choice in the diagnosis of gastrointestinal stromal tumours. *Histopathology* 2010; **57**:259-70.

Ou WB, Zhu MJ, Demetri GD, Fletcher CD, Fletcher JA. Protein kinase C-theta regulates KIT expression and proliferation in gastrointestinal stromal tumors. *Oncogene* 2008; **27**:5624-34.

Patel S. Exploring novel therapeutic targets in GIST: Focus on the PI3K/akt/mTOR pathway. *Curr Oncol Rep* 2013; **15**:386-95.

Quattrone A, Wozniak A, Dewaele B, Floris G, Vanspauwen V, Van LT, Schoffski P, Rutkowski P, Sciot R, Debiec-Rychter M. Frequent mono-allelic loss associated with deficient PTEN expression in imatinib-resistant gastrointestinal stromal tumors. *Mod Pathol* 2014;.

Rege TA, Wagner AJ, Corless CL, Heinrich MC, Hornick JL. "Pediatric-type" gastrointestinal stromal tumors in adults: Distinctive histology predicts genotype and clinical behavior. *Am J Surg Pathol* 2011; **35**:495-504.

Ricci R, Maggiano N, Castri F, Rinelli A, Murazio M, Pacelli F, Potenza AE, Vecchio FM, Larocca LM. Role of PTEN in gastrointestinal stromal tumor progression. *Arch Pathol Lab Med* 2004; **128**:421-5.

Rubin BP, Singer S, Tsao C, Duensing A, Lux ML, Ruiz R, Hibbard MK, Chen CJ, Xiao S, Tuveson DA, Demetri GD, Fletcher CD, Fletcher JA. KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. *Cancer Res* 2001; **61**:8118-21.

Samant RS, Clarke PA, Workman P. The expanding proteome of the molecular chaperone HSP90. *Cell Cycle* 2012; **11**:1301-8.

Schaefer IM, Delfs C, Cameron S, Gunawan B, Agaimy A, Ghadimi BM, Haller F. Chromosomal aberrations in primary PDGFRA-mutated gastrointestinal stromal tumors. *Hum Pathol* 2014; **45**:85-97.

Schaefer IM, Strobel P, Cameron S, Beham A, Otto C, Schildhaus HU, Agaimy A. Rhabdoid morphology in gastrointestinal stromal tumours (GISTs) is associated with PDGFRA mutations but does not imply aggressive behaviour. *Histopathology* 2014; **64**:421-30.

Schneider-Stock R, Boltze C, Lasota J, Miettinen M, Peters B, Pross M, Roessner A, Gunther T. High prognostic value of p16INK4 alterations in gastrointestinal stromal tumors. *J Clin Oncol* 2003; **21**:1688-97.

Schoffski P, Reichardt P, Blay JY, Dumez H, Morgan JA, Ray-Coquard I, Hollaender N, Jappe A, Demetri GD. A phase I-II study of everolimus (RAD001) in combination with imatinib in patients with imatinib-resistant gastrointestinal stromal tumors. *Ann Oncol* 2010; **21**:1990-8.

Scholl C, Frohling S, Dunn IF, Schinzel AC, Barbie DA, Kim SY, Silver SJ, Tamayo P, Wadlow RC, Ramaswamy S, Dohner K, Bullinger L, Sandy P, Boehm JS, Root DE, Jacks T, Hahn WC, Gilliland DG. Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. *Cell* 2009; **137**:821-34.

Schultz N, Marenstein DR, De Angelis DA, Wang WQ, Nelander S, Jacobsen A, Marks DS, Massague J, Sander C. Off-target effects dominate a large-scale RNAi screen for modulators of the TGF-beta pathway and reveal microRNA regulation of TGFBR2. *Silence* 2011; **2**:3,907X-2-3.

Serrano C, Wang Y, Marino-Enriquez A, Lee JC, Ravegnini G, Morgan JA, Bertagnolli MM, Beadling C, Demetri GD, Corless CL, Heinrich MC, Fletcher JA. KRAS and KIT gatekeeper mutations confer polyclonal primary imatinib resistance in GI stromal tumors: Relevance of concomitant phosphatidylinositol 3-kinase/AKT dysregulation. *J Clin Oncol* 2014;.

Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 2014; **343**:84-7.

Shao DD, Tsherniak A, Gopal S, Weir BA, Tamayo P, Stransky N, Schumacher SE, Zack TI, Beroukhim R, Garraway LA, Margolin AA, Root DE, Hahn WC, Mesirov JP. ATARiS: Computational quantification of gene suppression phenotypes from multisample RNAi screens. *Genome Res* 2013; **23**:665-78.

Sharma K, Vabulas RM, Macek B, Pinkert S, Cox J, Mann M, Hartl FU. Quantitative proteomics reveals that Hsp90 inhibition preferentially targets kinases and the DNA damage response. *Mol Cell Proteomics* 2012; **11**:M111.014654.

Sigoillot FD, Lyman S, Huckins JF, Adamson B, Chung E, Quattrochi B, King RW. A bioinformatics method identifies prominent off-targeted transcripts in RNAi screens. *Nat Methods* 2012; **9**:363-6.

Singh S, Wu X, Ljosa V, Bray MA, Piccioni F, Root DE, Doench JG, Boehm JS, Carpenter AE. Morphological profiles of RNAi-induced gene knockdown are highly reproducible but dominated by seed effects. *PLoS One* 2015; **10**:e0131370.

Smith JR, Clarke PA, de Billy E, Workman P. Silencing the cochaperone CDC37 destabilizes kinase clients and sensitizes cancer cells to HSP90 inhibitors. *Oncogene* 2009; **28**:157-69.

Smith JR, de Billy E, Hobbs S, Powers M, Prodromou C, Pearl L, Clarke PA, Workman P. Restricting direct interaction of CDC37 with HSP90 does not compromise chaperoning of client proteins. *Oncogene* 2015; **34**:15-26.

Smith JR, Workman P. Targeting CDC37: An alternative, kinase-directed strategy for disruption of oncogenic chaperoning. *Cell Cycle* 2009; **8**:362-72.

Sos ML, Fischer S, Ullrich R, Peifer M, Heuckmann JM, Koker M, Heynck S, Stuckrath I, Weiss J, Fischer F, Michel K, Goel A, Regales L, Politi KA, Perera S, Getlik M, Heukamp LC, Ansen S, Zander T, Beroukhim R, Kashkar H, Shokat KM, Sellers WR, Rauh D, Orr C, Hoeflich KP, Friedman L, Wong KK, Pao W, Thomas RK. Identifying genotype-dependent efficacy of single and combined PI3K- and MAPK-pathway inhibition in cancer. *Proc Natl Acad Sci U S A* 2009; **106**:18351-6.

Stratakis CA, Carney JA. The triad of paragangliomas, gastric stromal tumours and pulmonary chondromas (carney triad), and the dyad of paragangliomas and gastric stromal sarcomas (carney-stratakis syndrome): Molecular genetics and clinical implications. *J Intern Med* 2009; **266**:43-52.

Tabone-Eglinger S, Subra F, El SH, Alberti L, Tabone E, Michot JP, Theou-Anton N, Lemoine A, Blay JY, Emile JF. KIT mutations induce intracellular retention and activation of an immature form of the KIT protein in gastrointestinal stromal tumors. *Clin Cancer Res* 2008; **14**:2285-94.

Taguchi T, Sonobe H, Toyonaga S, Yamasaki I, Shuin T, Takano A, Araki K, Akimaru K, Yuri K. Conventional and molecular cytogenetic characterization of a new human cell line, GIST-T1, established from gastrointestinal stromal tumor. *Lab Invest* 2002; **82**:663-5.

Taipale M, Krykbaeva I, Koeva M, Kayatekin C, Westover KD, Karras GI, Lindquist S. Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. *Cell* 2012; **150**:987-1001.

Vaughan CK, Mollapour M, Smith JR, Truman A, Hu B, Good VM, Panaretou B, Neckers L, Clarke PA, Workman P, Piper PW, Prodromou C, Pearl LH. Hsp90-dependent activation of protein kinases is regulated by chaperone-targeted dephosphorylation of Cdc37. *Mol Cell* 2008; **31**:886-95.

Verweij J, Casali PG, Zalcberg J, LeCesne A, Reichardt P, Blay JY, Issels R, van Oosterom A, Hogendoorn PC, Van Glabbeke M, Bertulli R, Judson I. Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: Randomised trial. *Lancet* 2004; **364**:1127-34.

Wagner AJ, Chugh R, Rosen LS, Morgan JA, George S, Gordon M, Dunbar J, Normant E, Grayzel D, Demetri GD. A phase I study of the HSP90 inhibitor retaspimycin hydrochloride (IPI-504) in patients with gastrointestinal stromal tumors or soft-tissue sarcomas. *Clin Cancer Res* 2013; **19**:6020-9.

Wang CM, Huang K, Zhou Y, Du CY, Ye YW, Fu H, Zhou XY, Shi YQ. Molecular mechanisms of secondary imatinib resistance in patients with gastrointestinal stromal tumors. *J Cancer Res Clin Oncol* 2010; **136**:1065-71.

Wardelmann E, Hrychyk A, Merkelbach-Bruse S, Pauls K, Goldstein J, Hohenberger P, Losen I, Manegold C, Buttner R, Pietsch T. Association of platelet-derived growth factor receptor alpha mutations with gastric primary site and epithelioid or mixed cell morphology in gastrointestinal stromal tumors. *J Mol Diagn* 2004; **6**:197-204.

Wilson FH, Johannessen CM, Piccioni F, Tamayo P, Kim JW, Van Allen EM, Corsello SM, Capelletti M, Calles A, Butaney M, Sharifnia T, Gabriel SB, Mesirov JP, Hahn WC, Engelman JA, Meyerson M, Root DE, Janne PA, Garraway LA. A functional landscape of resistance to ALK inhibition in lung cancer. *Cancer Cell* 2015; **27**:397-408.

Workman P. Combinatorial attack on multistep oncogenesis by inhibiting the Hsp90 molecular chaperone. *Cancer Lett* 2004; **206**:149-57.

Xu W, Mollapour M, Prodromou C, Wang S, Scroggins BT, Palchick Z, Beebe K, Siderius M, Lee MJ, Couvillon A, Trepel JB, Miyata Y, Matts R, Neckers L. Dynamic tyrosine phosphorylation modulates cycling of the HSP90-P50(CDC37)-AHA1 chaperone machine. *Mol Cell* 2012; **47**:434-43.

Yang J, Ikezoe T, Nishioka C, Takezaki Y, Hanazaki K, Taguchi T, Yokoyama A. Long-term exposure of gastrointestinal stromal tumor cells to sunitinib induces epigenetic silencing of the PTEN gene. *Int J Cancer* 2012; **130**:959-66.

Yang X, Boehm JS, Yang X, Salehi-Ashtiani K, Hao T, Shen Y, Lubonja R, Thomas SR, Alkan O, Bhimdi T, Green TM, Johannessen CM, Silver SJ, Nguyen C, Murray RR, Hieronymus H, Balcha D, Fan C, Lin C, Ghamsari L, Vidal M, Hahn WC, Hill DE, Root DE. A public genome-scale lentiviral expression library of human ORFs. *Nat Methods* 2011; **8**:659-61.

Zhang T, Li Y, Yu Y, Zou P, Jiang Y, Sun D. Characterization of celastrol to inhibit HSP90 and CDC37 interaction. *J Biol Chem* 2009; **284**:35381-9.

Zhang Y, Schulz VP, Reed BD, Wang Z, Pan X, Mariani J, Euskirchen G, Snyder MP, Vaccarino FM, Ivanova N, Weissman SM, Szekely AM. Functional genomic screen of human stem cell differentiation reveals pathways involved in neurodevelopment and neurodegeneration. *Proc Natl Acad Sci U S A* 2013; **110**:12361-6.

Zhong R, Kim J, Kim HS, Kim M, Lum L, Levine B, Xiao G, White MA, Xie Y. Computational detection and suppression of sequence-specific off-target phenotypes from whole genome RNAi screens. *Nucleic Acids Res* 2014; **42**:8214-22.

Zhu MJ, Ou WB, Fletcher CD, Cohen PS, Demetri GD, Fletcher JA. KIT oncoprotein interactions in gastrointestinal stromal tumors: Therapeutic relevance. *Oncogene* 2007; **26**:6386-95.

Anexo

Publicaciones principales

I. Dystrophin is a tumor suppressor in human cancers with myogenic programs.

Wang Y, **Marino-Enriquez A**, Bennett RR, Zhu M, Shen Y, Eilers G, Lee JC, Henze J, Fletcher BS, Gu Z, Fox EA, Antonescu CR, Fletcher CD, Guo X, Raut CP, Demetri GD, van de Rijn M, Ordog T, Kunkel LM, Fletcher JA. *Dystrophin is a tumor suppressor in human cancers with myogenic programs.* Nature Genetics 2014;46:601-6.

II. *KRAS* and *KIT* gatekeeper mutations confer polyclonal primary imatinib resistance in GIST: relevance of concomitant PI3K/AKT dysregulation.

Serrano C, Wang Y, **Marino-Enriquez A**, Lee JC, Ravegnini G, Morgan JA, Bertagnolli MM, Beadling C, Demetri GD, Corless CL, Heinrich MC, Fletcher JA. *KRAS and KIT gatekeeper mutations confer polyclonal primary imatinib resistance in GIST: relevance of concomitant PI3K/AKT dysregulation.* Journal of Clinical Oncology 2014 Mar 31.

III. Sorafenib inhibits many kinase mutations associated with drug-resistant gastrointestinal stromal tumors.

Heinrich MC, **Marino-Enriquez A**, Presnell A, Donsky RS, Griffith DJ, McKinley A, Patterson J, Taguchi T, Liang CW, Fletcher JA. *Sorafenib inhibits many kinase mutations associated with drug-resistant gastrointestinal stromal tumors.* Molecular Cancer Therapeutics 2012;11:1770-80.

IV. Genome-wide functional screening identifies CDC37 as a crucial HSP90-cofactor for KIT oncogenic expression in gastrointestinal stromal tumors.

Marino-Enriquez A, Ou WB, Cowley G, Luo B, Jonker AH,Mayeda M, Okamoto M, Wang YX, Taguchi T, Demetri GD, Root DE, Fletcher JA. *Genome-wide functional screening identifies CDC37 as a crucial HSP90-cofactor for KIT oncogenic expression in gastrointestinal stromal tumors.* Oncogene 2014:33:1872-6.